Genomic approaches to understand the genetic response to *Phytophthora cinnamomi* Rands in *Castanea* spp.

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‘(...) o fruto dos frutos, o único que ao mesmo tempo alimenta e simboliza, cai de umas árvores altas, imensas, centenárias, que, puras como vestais, parecem encarnar a virgindade da própria paisagem.

Só em Novembro as agita uma inquietação funda, dolorosa, que as faz lançar ao chão lágrimas (...). Abrindo-as, essas lágrimas eriçadas de espinhos deixam ver numa camada fofa a maravilha singular de que falo, tão desafectada que até no próprio nome é doce e modesta – a **castanha**.’

Miguel Torga (‘Reino Maravilhoso’)

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List of abbreviations

µg	Microgram
µl	Microliter
µM	Micromolar
µmol	Micromole
^{o}C	Celsius degrees
A	Adenine
ABA	Abcisic acid
Bp	Base pair
BP	Biological process
C	Cytosine
Cc	*Castanea crenata*
CC	Cellular component
Cs	*Castanea sativa*
cDNA	Complementary DNA
cM	Centimorgan
Cm	*Castanea mollissima*
Cn/µL	Copy number per microliter
Cp	*Cryphonectria parasitica*
dai	Days after inoculation
DEGs	Diferentially expressed gene
DNA	Deoxyribonucleic acid
dPCR	Digital PCR
EST	Expressed sequence tag
ETI	Effector-triggered immunity
G	Guanine
GO	Gene ontology
He	Expected heterozygosity
Ho	Observed heterozygosity
hpi	Hours post inoculation
HR	Hypersensitive response
i	Inoculated
JA	Jasmonic acid
LG	Linkage group
LL	Lesion lenght
LOD	Logarithm of the odds
LRR	Leucine rich repeat

m	Meter
M	Molar
MAS	Market assisted selection
MF	Molecular function
min	Minutes
Mm	Milimolar
mol	Mole
mRNA	Messenger RNA
MS	Murashige and Skoog
ni	Non-inoculated
ng	Nanogram
NGS	Next generation sequencing
nt	Nucleotide
PAMP	Pathogen-associated molecular patterns
Pc	*Phytophthora cinnamomi*
PCR	Polymerase chain reaction
PPR	Pattern-recognition receptor
PTI	PAMP-triggered immunity
qRT-PCR	Quantitative real-time PCR
QS3D	Quantstudio^{tm} 3D Digital PCR System
QTL	Quantitative trait locus
RLK	Receptor-like kinase
RNA	Ribonucleic acid
RNA-Seq	Rna sequencing
SA	Salicylic acid
SE	Standard error
SC	*Castanea sativa* x *Castanea crenata* hybrid
SM	*Castanea sativa* x *Castanea mollissima* hybrid
SNP	Single nucleotide polimorphism
SSR	Simple sequence reapeat
T	Thymine
TACF	The American chestnut foundation
TF	Transcription factor

Summary

Chestnut is a multipurpose tree, having important economic, ecological and scientific values. European chestnut (*Castanea sativa*) produces the most appreciated and valued nuts worldwide. However, chestnut orchards and forests are declining in Europe due to introduced diseases and pests, mainly the ink disease. This destructive disease is caused by the widespread soil-borne oomycete *Phytophthora cinnamomi*. *P. cinnamomi* infection occurs in roots causing root rot and dieback in susceptible species. Nevertheless, the susceptible level varies among chestnut species, being the Asian species the most resistant to the pathogen.

The research work of this thesis was performed in progenies that segregate for the trait of interest, obtained from the Portuguese breeding program, based on controlled crosses between the resistant Japanese chestnut and the susceptible European chestnut (Portuguese cultivars) established 10 years ago. Mapping and transcriptomic approaches have been implemented aiming to unveil the different mechanisms of disease response across chestnuts. So far, 155 progenies were obtained, genotyped and phenotyped in order to map genomic regions controlling *P. cinnamomi* resistance (Quantitative Trait Loci-QTLs).

Using transcriptome-derived microsatellite and single nucleotide polymorphism (SNPs) as molecular markers, genotypic data was collected for the parental individuals and progenies. The segregation data was analysed for the construction of European x Japanese chestnut genetic map. The interspecific map contains 283 molecular markers, mapped on 15 linkage groups and spanning a total of 714.8 cM, which corresponds to about 96% of the Chinese chestnut reference map.

Phenotyping was performed by evaluating different disease metrics following *P. cinnamomi* inoculation of roots and/or excised shoots of mother plants. The lesion progression rate observed in the excised shoot inoculation

assays, was strongly and negatively correlated with the days of survival recorded after root inoculations. Therefore, the excised shoot inoculation test revealed be a reliable approach for screening the metrics of resistance of chestnut genotypes to *P. cinnamomi*. Moreover, a set of resistant genotypes was selected, constituting a valuable source of new genetic resources, essential to address the shortcomings of the Portuguese and European chestnut market.

The association between genotype and phenotype enabled the identification of unique QTLs for *P. cinnamomi* resistance. Ten QTLs were mapped on five linkage groups of the European x Japanese chestnut map. The presence of QTLs on linkage group E was consistent with a previous pilot study for identification of QTLs in backcross families (Chinese chestnut x American chestnut hybrid), suggesting that different *Castanea* species might share resistant haplotypes, and therefore, common resistance mechanisms.

Concerning the transcriptomic approach, candidate genes for *P. cinnamomi* resistance were identified from the root transcriptome of European and Japanese chestnut inoculated and non-inoculated with the pathogen. Those genes are involved, in both species, in the regulation of plant immune response and stress adaptation and recovery. The expression levels of eight of the candidate genes were quantified by digital PCR, using European and Japanese chestnut and four hybrid genotypes showing different levels of susceptibility to the disease. RNA-seq and gene expression analysis suggested that both species recognize the pathogen attack, which may trigger resistance signaling pathways and cell wall modification, as well as, the production of anti-fungal proteins. However, the resistant species may involve basal defense mechanisms, being protected in advance to the infection.

Additionally, new molecular markers were developed from the sequences of candidate genes identified by transcriptome sequencing. Forty-one microsatellite showing polymorphism and high transferability within and

among chestnut species were used for genotyping European x Japanese chestnut populations. Two of them were mapped within the identified QTL intervals, being strong candidates for further validation and marker-assisted selection.

The knowledge acquired in this project is a major breakthrough in understanding the *Castanea-P. cinnamomi* interactions and may contribute for the development of strategies to control ink disease. Furthermore, this project developed a crucial deliverable for farmers and society, since the genotypes with improved resistance to the pathogen have been propagated, to be released to the market as rootstocks, in the near future.

Sumário

O castanheiro é uma árvore polivalente, com importante impacto económico, ecológico e científico. O castanheiro Europeu (*Castanea sativa*) produz as castanhas mais apreciadas e valorizadas no mundo. No entanto, a área de soutos e castinçais está a diminuir na Europa devido a doenças e pragas, principalmente a doença da tinta. Esta doença altamente destrutiva é causada pelo oomiceta *Phytophthora cinnamomi,* difundido por todo o mundo. A infecção por *P. cinnamomi* ocorre nas raízes causando a sua podridão e levando à morte em espécies susceptíveis. No entanto, o nível susceptibilidade varia entre castanheiros, sendo as espécies asiáticas as mais resistentes ao patógeno.

A investigação desenvolvida durante esta tese foi realizada em descendências que segregam para a característica de interesse, obtidas a partir do programa de melhoramento estabelecido há 10 anos. Este programa é baseado em cruzamentos controlados entre o castanheiro japonês resistente e o castanheiro europeu susceptível ao agente patogénico. Foram implementadas abordagens de mapeamento e de transcriptómica visando compreender os diferentes mecanismos de resposta do castanheiro à doença. Até ao momento foram obtidas 155 descendências, que foram genotipadas e fenotipadas de forma a mapear as regiões genómicas que controlam a resistência a *P. cinnamomi* (*Quantitative Trait Loci-QTLs*).

Usando marcadores moleculares (microsatélites e SNPs) derivados de transcriptomas obtidos, previamente e durante este trabalho, foi realizada a genotipagem dos progenitores e respectivas descendências. Os dados de segregação obtidos foram analisados para a construção do primeiro mapa genético de castanheiro Europeu x castanheiro Japonês. O mapa genético interespecífico contém 283 marcadores moleculares, mapeados em 15

grupos de ligação e abrangendo um total de 714,8 cM, o que corresponde a cerca de 96% do mapa de referência de castanheiro Chinês.

A fenotipagem foi realizada através da avaliação das métricas de resposta à doença, obtidas para todas as descendências, após a inoculação de raízes e/ou de estacas excisadas das plantas-mãe, com *P. cinnamomi*. A taxa de progressão da lesão observada nos ensaios de inoculação realizados em estacas foi fortemente e negativamente correlacionada com os dias de sobrevivência registados após a inoculação das raízes. Assim, o teste de inoculação em estaca demonstrou possuir rigor para avaliar a resistência à doença da tinta em diferentes genótipos de castanheiro. Além disso, foram selecionados um conjunto de genótipos com resistência melhorada a *P. cinnamomi*, constituindo novos recursos genéticos essenciais para colmatar o elevado défice de material vegetal melhorado no mercado tanto em Portugal e na Europa.

A associação entre genótipo e fenótipo permitiu a identificação de QTLs relacionados com a resistência a *P. cinnamomi* pela primeira vez em castanheiro. Dez QTLs foram mapeados em cinco grupos de ligação do mapa genético de castanheiro Europeu x castanheiro Japonês. A presença de QTLs em determinados grupos de ligação foi consistente com um estudo piloto realizado anteriormente para a identificação de QTLs em famílias do programa de melhoramento Americano, sugerindo que as diferentes espécies do género *Castanea* podem partilhar haplótipos e mecanismos de resistência.

Em relação à abordagem de transcriptómica, os genes candidatos para a resistência a *P. cinnamomi* foram seleccionados a partir do transcriptoma de raízes de castanheiro europeu e japonês respetivamente inoculadas e não inoculadas com o patogénio. Para ambas as espécies, estes genes estão envolvidos na regulação da resposta imune das plantas e na adaptação e recuperação do stress biótico. Os níveis de expressão de oito genes foram quantificados por PCR digital, em raízes de castanheiro Europeu e Japonês

e de quatro genótipos híbridos mostrando diferentes níveis de susceptibilidade à doença. A análise de expressão génica e da sequenciação do transcriptoma, sugere que ambas as espécies reconhecem o ataque de patogénio, podendo desencadear vias de sinalização de resistência que podem resultar na a modificação da parede celular e/ou na produção de proteínas antifúngicas. No entanto, as espécies resistentes parecem envolver mecanismos de defesa basal, encontrando-se protegidas antecipadamente à infecção.

Adicionalmente, foram desenvolvidos novos marcadores moleculares a partir das sequências de genes candidatos, identificados na sequenciação dos transcriptomas. Quarenta e um microssatélites mostrando polimorfismo e alta transferibilidade, dentro e entre as diferentes espécies de castanheiro, foram utilizados para a genotipagem das populações híbridas de castanheiro Europeu x castanheiro Japonês. Dois dos marcadores desenvolvidos foram mapeados dentro de intervalos dos QTLs identificados, por isso constituem-se como fortes candidatos para validação adicional e seleção assistida por marcadores moleculares.

O conhecimento adquirido neste estudo constituiu um grande avanço na compreensão da interação entre *Castanea* e *P. cinnamomi*, podendo contribuir para o desenvolvimento de estratégias de controlo da doença da tinta. Além disso, este projecto desenvolveu um valioso produto para os agricultores e para a sociedade, uma vez que os genótipos com resistência melhorada ao patógenio que estão a ser propagados, serão lançados no mercado, como os porta-enxertos, num futuro próximo.

Chapter I

General Introduction

The chestnut: since ancient times to the present

The chestnut is a multipurpose tree that has a very ancient history and tradition, as well as an important economic and environmental role. Chestnuts were historically distributed only throughout the northern hemisphere, but due to anthropogenic influences have been introduced into Chile, Argentina, Australia and New Zealand, being currently widely cultivated all over the temperate regions (Conedera and Krebs, 2008; Pereira-Lorenzo et al. 2012).

Since the Middle Ages, the nuts of European chestnut, a noble hardwood, and also of Japanese and Chinese chestnuts, provided an essential food source, resulting in diverse types of use: fresh consumption, long-term storage, drying, flour and animal feed (Bounous and Marinoni 2005; Bounous 2009). In North America, the American chestnut, known as a forest giant, was a dominant species along the Appalachian range. The American chestnut trees played a dominant role for American people, since the wood was extensively used for building houses and furniture or used as fuelwood. Nuts were part of the human diet and also, they had an important role for wildlife in the forests (Anagnostakis, 2012; Jacobs et al. 2015).

Nowadays, the chestnut continues to have an important role in many agroforestry systems. Although they are no longer a subsistence food, chestnuts are currently an appreciated product for an increasingly large market sector. The nuts, with both modern and traditional methods of storage and processing, meet the demand of consumers, who are progressively seeking for nutritious and healthy foods.

Impact of chestnut in economy and environment

The cultivation of chestnut has been mainly related with the abundant and versatile uses of the products that can be obtained: edible nuts, timber for building and for other woody products, associated mushrooms production and extracted tannins for tanning leather or pharmaceutical purposes.

Moreover, the chestnut ecosystems always contributed for biodiversity preservation, representing an important cultural heritage (Paillet 2002; Bounous 2005; Bounous and Marinoni 2005; Conedera and Krebs 2008; Bounous 2009).

Chestnuts are one of the most important nut crops in the temperate zone. They have a delicious taste, being consumed in fresh, dried or processed. Processing is necessary to increase the available products and to extend the use of the product along the year (Bounous and Marinoni 2005). They have many culinary uses, ranging from first to main course dishes (used as side or served whole, boiled or roasted) as well as vegetable dishes (as soups, chestnut purées), desserts (marrons glacés, cakes, ice-creams and syrups) and pastries (as chestnut creams, mousse). It is also possible to prepare beverages from chestnuts such as liqueurs, beers and non-alcoholic drinks. Some examples of chestnut uses are shown in Figure 1. From a nutritional point of view, chestnuts are a very healthy, balanced and high-energy food. Fresh chestnuts are high in calorie content, low in fat and sodium, free of cholesterol and gluten, with a moderate but high-quality protein content and a favorable amino-acid ratio (Pereira-Lorenzo et al. 2006; De Vasconcelos et al. 2010).

Many countries around the world have suitable edaphoclimatic conditions for chestnut plantation. The European, Chinese and Japanese chestnuts are the main species cultivated for fruit, due to their large nut size. Nevertheless, European chestnut (*marrone* types) are considered the most valuable for nut production (Pereira-Lorenzo et al. 2012). Interspecific hybrids which have emerged from disease resistance studies are also used for nut production directly or as rootstocks.

Figure 1. Chestnut multiuses. (A) Fresh chestnuts; (B) Roasted chestnuts; (C) Marron glacés; (D) Chestnut flour; (E) Chestnut liqueur; (F) Chestnuts in syrup; (G) Chestnut cake and (H) Mushrooms production associated to chestnut orchards. Photographs by José Gomes-Laranjo (A-C), Rita Costa (D), Carmen Santos (E and G), Sweet *Castanea* (F) and Helena Machado (H).

Although production in East Asian is increasing, new orchards are being established in Europe, North and South America and Australia, due to the high demand for high quality nuts (Bounous, 2009). According to FAO statistics there were 544 453 ha of chestnut orchards in the world in 2013 and chestnut production exceeded 2 million tones, with China accounting for more than 80% of the total. Korea was the second best producer, harvesting about 70 000 tones per year. In Europe, Turkey and Italy are the leading producers, followed by Portugal and Spain (Table 1). The total European harvest area corresponds to 109 535 ha, from which 35 168 ha are located in Portugal. Chestnuts are the most exported fruit in Portugal, corresponding to 13 000 tonnes per year and profiting more than 30 million € annually (Instituto Nacional de Estatística, Portugal). Other worldwide great producers are Chile, Bolivia, the USA, France and Greece.

Table 1. Chestnut production trends 2009-2013 worldwide and specifically for the main Asian and European chestnut producers: China, Korea, Japan, Turkey, Italy, Portugal and Spain (FAOSTAT, 2016, faostat.fao.org). Values are presented in tones per year, per country.

Country	2009	2010	2011	2012	2013
China	1 550 000	1 620 000	1 600 000	1 650 000	1 650 000
Korea	75 911	68 630	64 586	70 000	67 902
Japan	21 700	23 500	19 100	20 900	21 000
Turkey	61 697	59 171	60 270	57 881	60 019
Italy	50 872	48 810	50 134	52 000	49 459
Portugal	24 305	22 350	18 271	19 100	24 700
Spain	16 000	17 900	16 900	15 300	17 200
Others	98 770	104 237	105 971	117 629	119 207
Total	**1 899 255**	**1 964 598**	**1 935 232**	**2 002 810**	**2 009 487**

Chestnut wood has been valued because it is easy to work with, straight-grained, lightweight, durable, rot-resistant and rich in tannin. Nowadays, the economic impact of chestnut timber in Europe has decreased, since orchards for nut production has been preferred. However in Europe chestnut timber was used for many purposes, such as furniture, building and fuel wood (Bounous and Marinoni 2005). Likewise, before the American chestnut devastation in the USA, wood from this tree was abundant and used for construction of buildings and furniture, shipbuilding and musical instruments (Anagnostakis and Hillman 1992; Anagnostakis 2001).

An important chestnut economic resource is the associated edible mushroom production. Chestnut root system is strong, expanded and penetrates the soil deeply; the thinnest roots are abundantly in symbiosis with ectomycorrhizal fungi. Mushrooms of high gastronomic interest, like some species of *Boletus* (porcini) and other genus, are important chestnut by-products (Peintner et al. 2007) (Figure 1.H).

Beyond the economic impact, chestnut trees still characterize the landscape in several native regions, with its unique botanical features such as the

serrated leaves and the singular flowers and fruits. As part of the ecosystems, chestnuts have an important ecological role in avoiding fire progression, soil fixation and microbe diversity and also provide a food resource for wildlife, insects and livestock (Bounous and Marinoni, 2005; Jacobs et al. 2015; Paillet, 2002; Rebelo, 2016).

The *Castanea* genus

Taxonomy, diversity and distribution

Chestnuts are deciduous long-living species taxonomically belonging to the *Castanea* genus and Fagaceae family. Besides the *Castanea* genus, the Fagaceae comprises 8-10 genera, which includes important timber producing trees, such as *Quercus* (oaks) and *Fagus* (beech). The most Fagaceae species studied to date, are diploid with haploid (n) numbers of 12 chromosomes (2n=24). Altogether, Fagaceae family includes about a thousand tree species, mainly distributed through the Northern hemisphere (Kremer et al. 2012).

Castanea is highly variable for morphological and ecological traits, vegetative and reproductive habits, nut size, wood characteristics, adaptability and resistance to biotic and abiotic stresses. Therefore, several useful morphological traits have been used to identify *Castanea* species and hybrids (Elorrieta 1949; Martin et al. 2009; Dinis et al. 2011; González et al. 2011; Fernández-Cruz and Fernández-López 2012; Mellano et al. 2012; Marinoni et al. 2013). However, the classification and differentiation of species and hybrids is not straightforward because many traits are shared or overlapped.

The biogeographical history of the genus has been inferred from analysis of chloroplastidial DNA, suggesting that the chestnut was originated in eastern Asia (Japan), following an intercontinental dispersion and divergence between the Chinese and European/North American species. A subsequent

divergence occurred between the European and North American species (Lang et al. 2007).

Castanea genus includes 13 species and is taxonomically divided into 3 sections (Table 2): *Eucastanon* (chestnuts), *Balanocastanon* (chinkapins), and *Hypocastanon* (henryi chestnuts). The section *Eucastanon* is characterized by three nuts per cupule and display high genetic diversity, comprising the species with major economic and ecological importance: European chestnut (*C. sativa* Miller), American chestnut (*C. dentata* Borkhausen), Chinese chestnut (*C. mollissima* Blume) and Japanese chestnut (*C. crenata* Sieb. & Zucc.) (Mellano et al. 2012).

Table 2. Origin, taxonomy and prevalent uses of *Castanea* species (adapted from Mellano et al. 2012).

Origin	Section	Species	Common name	Main use
Europe	*Eucastanon*	*C. sativa*	European chestnut or sweet chestnut	Nut, timber
Asia		*C. crenata*	Japanese chestnut	Nut
		C. mollissima	Chinese chestnut	Nut
		C. seguinii	-	Firewood
		C. davidii	-	Firewood
	Hypocastanon	*C. henryi*	Willow leaf or pearl chestnut	Timber
America	*Eucastanon*	*C. dentata*	American chestnut	Timber
	Balanocastanon	*C. pumila* var. *pumila*	Allegheny chinkapin	Nut
		C. pumila var. *ozarkensis*	Ozark chinkapin	Timber
		C. floridana	Florida chinkapin	Ornamental
		C. ashei	Ashe chinkapin	Ornamental
		C. alnifolia	Creeping chinkapin	-
		C. paucispina	-	-

The natural range of *Castanea* species has been extended worldwide, especially in the mountainous regions (Figure 2). *C. sativa* is predominant in all Mediterranean countries: Turkey, Greece, Slovenia, Italy, Germany, France, Spain, Portugal and Southern England. It is also found in small areas bordering North Africa: Morocco, Algeria and Tunisia (Konstantinidis et al. 2008).

C. dentata is native to the eastern United States and Canada and it was once a long dominant species, covering more than 200 million acres from Ontario and Maine and along the Appalachian Mountain range into Georgia and Alabama (Russell 1987).

C. mollissima is an important native species in China, found in both wild and cultivated stands. *C. mollissima* grows in subtropical, temperate-continental, and temperate-maritime regions with mild winters and hot summers (Bounous and Marinoni 2005; Pereira-Lorenzo et al. 2012).

C. crenata is naturally distributed and cultivated in the Korean Peninsula, Japan and the temperate region of East Asia.

Chestnut species and hybrids have been introduced in other regions, where climatic conditions are suitable (Figure 2). For example, *C. sativa* was introduced into Chile by European settlers at the beginning of the nineteenth century (Pereira-Lorenzo et al. 2012).

Chestnuts are generally adapted to deep, soft, high drainage, acidic soils (pH ranging from 4 to 6.5), rich in phosphorus and potassium. Deep soil and a deep root system are important to help trees maintain their water potential during the dry hot summer months (June to September) (Martins et al. 2005). They are commonly grown in poor sandy to loamy soil on slopes but also in volcanic islands (Sicily, Canary, Madeira and Azores). Chestnuts prefer temperate climates (average temperature is 8-15°C, but temperatures of 27º-30°C are required for blossoming and pollination), and rainfall ranging from 700 to 1500 mm/year. The latitude distribution is related to altitude.

Nevertheless, the different species reflect adaptation to different pedoclimates (Bounous and Marinoni 2005).

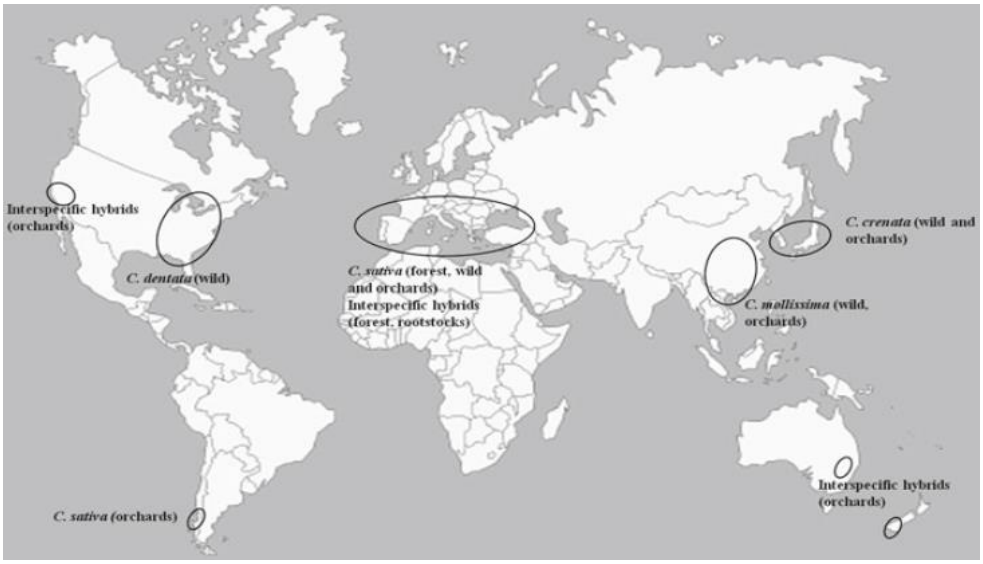

Figure 2. Actual chestnut distribution (Pereira-Lorenzo et al. 2012).

The European chestnut

European chestnut (*Castanea sativa* Miller) is a vigorous upright tree of majestic appearance. It can exceed 30 m in height, 10 m in girth and could live up to 1 000 years of age. Among the most important features of *C. sativa* is large nut size, with high density and sweet taste. An advantage of cultivating *C. sativa* trees was their low maintenance; harvesting is the costliest aspect of chestnut production (Conedera and Krebs 2008; Pereira-Lorenzo et al. 2012).

Before last glaciation, there were two taxa of chestnut in Europe: *C. sativa* and *C. latifolia* Sord (Paganelli A. 1997), however only *C. sativa* survived, being now the only native species in Mediterranean and Central European regions.

The first unambiguous pollen data showing evidence of European chestnut trees spreading due to human activities date back to around 2100-2050 B.C. (Conedera et al. 2004). Currently, *C. sativa* is commonly found in Europe between 400 and 1 000 m above sea level depending on the latitude. The lowest elevations are recommended for the highest latitudes and vice versa (Mellano et al. 2012).

The European chestnut germplasm is very extensive; there are hundreds of cultivar names used for chestnuts, many of which are synonyms or homonyms (Botta et al. 2001). In 2013, Italy was the largest chestnut producer in the European Union (FAOSTAT, 2016, faostat.fao.org), and led the world in producing processed chestnut products such as *marron glacé* (Bounous 2009). However, the introduction of gall wasp has decreased the nut production (Battisti et al. 2014). Italian *marron* type cultivars are 'Chiusa Pesio', 'Luserna', 'Val Susa', 'Castel del Rio', 'Marradi' and 'Fiorentino'.

Within Europe, France is the largest chestnut importer, mostly buying from Italy, but also from Spain and Portugal. Some *C. sativa* traditional French cultivars include 'Bouche Rouge', 'Verdale', 'Arizinca', 'Toumive', 'Belle Epine', 'Savoye', 'Châtaigne de Laguepie', 'Sardonne', 'Rouse de Nay', and 'Dorée de Lyon' (Mellano et al. 2012).

In Iberian Peninsula, it seems that cultivar diversification was a result of distinct genotypes being related via hybridization and mutation, regardless of whether they shared the same name or not (Pereira-Lorenzo et al. 2011). In Spain, the main cultivars are 'Loura', 'Garrida', and 'Parede', being 'Garrida' more suited to industrial purposes, conservation and genetic studies and European breeding programs (Pereira-Lorenzo et al. 2001; Blanco Silva and Fernández-López, 2005; Pereira-Lorenzo et al. 2006; Míguez-Soto and Fernández-López 2012; Fernández-López and Fernández-Cruz, 2015; Míguez-Soto and Fernández-López 2015; Fernández-Cruz and Fernández-López, 2016).

In Portugal, more than 25 cultivars are known, they showed great genetic variability (each cultivar includes different genotypes) indicating their polyclonal origin (Costa et al. 2008). One of the most ancestral cultivar, that is distributed throughout the entire Iberian Peninsula, is 'Longal' that has been promoted as the best cultivar for industry (Pereira-Lorenzo et al. 2011). 'Judia' and 'Martaínha', due to their larger nut size, are usually preferred for the fresh market.

In Portugal, chestnut is distributed mainly in the Northeast (*Trás-os-Montes*, *Minho* and *Beira Litoral*) but also is found in the center east, especially in *Marvão* region (Costa et al. 2008). Accordingly, four regions of Protected Designation of Origin (POD) were created to preserve the Portuguese cultivars: *Castanha da Terra Fria*, *Castanha da Padrela*, *Castanha dos Soutos da Lapa* and *Castanha do Marvão*.

Reproductive biology and hybridization

Castanea species is a monoecious species that generally flowers from June to July depending on the species; Asian species show precocious blossoming when compared to European species (Botta et al. 1995). *C. sativa* have been reported to begin flowering after 8-10 years, but flowering time can be shorted though grafting. Production is regular and high (Gomes-Laranjo et al. 2009).

Female flowers are pollinated by wind (more usual in case of dry weather during flowering) or insects (dominating in wet weather conditions). Inflorescence male flowers are gathered in catkins that can occur in two types: bisexual catkins that bear one or more female flowers at the base and male flowers toward the tip; and unisexual male catkins, also called staminate catkins (Mert and Soylu 2006). Female inflorescence generally contains three flowers that are protected by a green, scaled wrapping that is destined to form the cupule that develops into the chestnut bur. Usually female inflorescences are positioned at the base of the male ones, in the

upper part of the current year's shoots (Hebard et al. 2014a). Fertilization produces typically three large and brownish nuts encapsulated in a spiny bur. The burs protect the seeds until they are ripe and then open widely, making the nuts readily available. Nuts ripen early in September to November (Hebard et al. 2014a). The nuts of *Castanea* genus vary greatly among species and cultivars. Nevertheless, in average European chestnuts show the largest size in the genus and can weight more than 30 g (Figure 3). Chinese and Japanese chestnuts have similar size and the American chestnuts are much smaller (Figure 3).

Figure 3. Chestnuts morphology depending on the species. Photograph by Dr. Paul Sisco.

The chestnut is very often self-incompatible, therefore cross-pollination is compulsory (Mert and Soylu 2006). Very little is still known about the genetic system controlling mating and the self-incompatibility system in chestnut, although it is considered to be of gametophytic type (Zou et al. 2014).

Interspecific hybridizations between all *Castanea* species are possible. Main problems are related with the different flowering time between species. In all interspecies crosses, chromosome pairing exists among *Castanea* species, but the presence of segregation distortion in some mapping populations (Casasoli et al. 2001; Kubisiak et al. 1997; Kubisiak et al. 2013) suggest that significant chromosomal differences such as translocations and/or inversions may occur.

For breeding purposes, artificial controlled crosses have been performed (Costa et al. 2011; Takada et al. 2012; Nishio et al. 2013; Nelson et al. 2014; Fernández-Cruz 2015): donor pollen is easily collected from the catkins; receptor female flowers are isolated avoiding unknown pollination; male flowers from the receptor tree must be also removed (emasculation); manual pollination is performed by placing donor pollen over the pistils; pollination

bags are used for covering female flowers avoiding pollen contamination; pollination bags are removed and replaced by net bags in order to collect the burs containing the nuts.

Genomic resources

Genomic research on forest trees has been motivated by the need to support genetic breeding programs and develop tools for conservation, restoration and management of natural populations (Neale and Kremer 2011). Important genomic resources such as ESTs molecular markers and genetic maps have been developed for chestnut and integrated in a Web-based resource for the *Castanea* genetics/genomics community (Fagaceae Genomic Database: www.fagaceae.org). Moreover, the whole genome sequencing project for *C. mollissima* is underway (www.hardwoodgenomics.org). Based on flow cytometric analysis, genome size appears to be fairly conserved among species: the estimated 1C genome size of *C. sativa* is 777Mb, of *C. crenata* and *C. mollissima* is 794 Mb and of *C. dentata* is 803 Mb (Kremer et al. 2007). Therefore, the genome size of *Castanea* species is only five times larger than *Arabidopsis* and less than twice the size of the poplar genome. The manageable genome size and abundant genetic and genomic resources make *Castanea* a good candidate as model for Fagaceae family in the near future.

A large component of chestnut genomic resources is focused on the transcriptomes obtained for *C. mollissima*, *C. dentata*, *C. sativa* and *C. crenata* (Barakat et al. 2009; Sebastiana et al. 2009; Nishio et al. 2011; Barakat et al. 2012; Serrazina et al. 2015, Chapter III). Large EST databases are being created with significant numbers of sequence contigs showing similarity to predicted proteins in woody plants. Resistance candidate genes to chestnut blight and ink disease (Barakat et al. 2009; Barakat et al. 2012; Serrazina et al. 2015, Chapter III). have been identified in EST sequence data, as well as candidate genes for other traits (Sebastiana et al. 2009;

Nishio et al. 2011). Furthermore, a great number of molecular markers have been developed from those sequences mainly Simple Sequence Repeats (SSRs) or microsatellites and Single Nucleotide Polymorphism (SNPs) (Nishio et al. 2011; Kubisiak et al. 2013; Santos et al. 2015, Chapter V). Beyond molecular markers derived from ESTs databases, smaller sets of SSR markers were earlier developed from enriched genomic libraries of European chestnut (Marinoni et al. 2003; Buck et al. 2003), Japanese chestnut (Yamamoto et al. 2003) and Chinese chestnut (Inoue et al. 2009). Microsatellite and SNP markers are highly informative, transferable across related taxa, having great prevalence in the genome and amenability to automated high-throughput analysis. Therefore, the molecular markers developed so far for chestnut are an invaluable resource for the scientific community interested in all aspects of the genetics, breeding and biotechnology. For breeding purposes, the molecular markers have been mapped on genetic maps constructed for the four main *Castanea* species (Kubisiak et al. 1997; Casasoli et al. 2001; Sisco et al. 2005; Kubisiak et al. 2013; Nishio et al. 2013 and Chapter V). The genetic map constructed for *C. mollissima* (Kubisiak et al. 2013) was accepted as the chestnut reference map and was integrated with the physical map obtained by sequencing of BAC libraries (Fang et al. 2013). Quantitative trait loci (QTLs) related with chestnut blight and adaptive traits have been identified and confirmed (Kubisiak et al. 1997; Casasoli et al. 2004; Kubisiak et al. 2013). Moreover, syntenic regions have been identified between the chestnut physical map and some genomes available of other related taxa, revealing syntenic regions between QTLs for reistance to chestnut blight diasese and QTLs for resistance to other fungal pathogens in *Prunus* spp. (Staton et al. 2015). However, only an exploratory study identified QTLs for *P. cinnamomi* resistance (Zhebentyayeva et al. 2014) until to the present study.

Major diseases affecting chestnut

The most damaging diseases of chestnut are the ink disease, caused by the oomycete *Phytophthora cinnamomi* (and *P. cambivora*) and chestnut blight caused by the ascomycete fungus *Chryphonectria parasitica.* European and the American chestnut are highly susceptible to these pathogens whereas, Asian *Castanea* species show great resistance to the diseases (Crandall et al. 1945).

Ink disease

Ink disease, also known as root rot, is the most destructive disease affecting European chestnut. In most cases, *Phytophthora cinnamomi* is the causal agent of ink disease, whilst *Phytophthora cambivora* is less frequent and aggressive (Gouveia 2004). *P. cinnamomi* is a soilborne pathogen that parasites fine roots causing root and collar rot that extends to trunk and branches of young and mature trees, and consequently causes death. Typical symptoms include chlorosis and wilting of foliage, dieback of branches and crown (Robin et al. 2001; Vettraino et al. 2001; Hardham 2005; Kamoun et al. 2014). It is a silent disease since when first symptoms become visible in the crown the destruction of the fine root system is already in an advanced stage.

The geographical origin of *P. cinnamomi* is not clearly established, however there are evidences for an Asian origin and it was spread across the Pacific to Latin America (Ko et al. 1978; Zentmyer 1988; Zhang et al. 1994). Ink disease on *Castanea* was first reported in Portugal in 1838 (Vettraino et al. 2001), and in the USA in 1825 (Rhoades et al. 2003). Since then, *P. cinnamomi* have spread over Europe and North America and their native plant species were not adapted and therefore they are often highly susceptible. Currently, *P. cinnamomi* is the most widely distributed *Phytophthora* species (Figure 4). Furthermore, climate change is predicted to have a significant impact on the intensity and distribution of *P. cinnamomi*

(Thompson et al. 2014). The pathogen infects more than 3000 host species causing great economic impacts in forestry and horticulture, and in the nursery industry (Hardham 2005). Beyond *Castanea* species, *P. cinnamomi* affects most of the temperate of fruit trees such as *Persea, Quercus*, *Ericaceae*, *Eucalyptus*, *Cinnamomum*, *Coniferales*, *Fagus*, *Juglans* and many ornamental trees and shrubs (Hardham 2005; Robin et al. 2012).
Like other *Phytophthora* spp., *P. cinnamomi* has a number of strategies for survival, propagation and dissemination. It is an oomycete and not a fungus, although everything about its biology and life cycle is fungus-like, such as mycelial growth habit. Features that differ oomycetes from fungi include the production of biflagellate heterokont zoospores, the occurrence of cellulose rather than chitin in the cell walls and diploid somatic cells (Hardham et al. 1994; Hardham 2005).

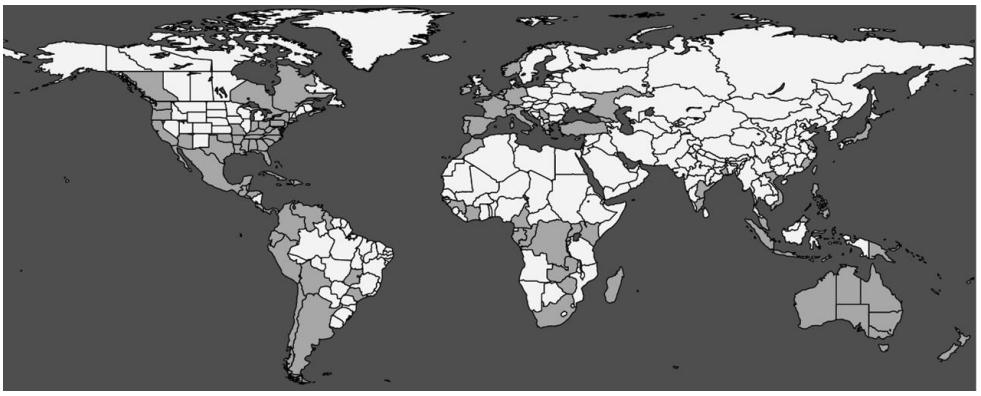

Figure 4. *Phytophthora cinnamomi* distribution worldwide (in grey), adapted from European and Mediterranean Plant Protection Organization, Global Database (2016).

P. cinnamomi is able to survive under unsuitable environmental conditions over several years in the soil or in infected root tissue, as dormant resting spores: chlamydospores, which are the most common or oospores, produced when different strains mate. Although sexual reproduction of *P. cinnamomi* is poorly understood, it is known that the pathogen is

heterothallic, requiring the presence of opposite mating types, designated A1 and A2, to form oospores (Hüberli et al. 1997; Hardham 2005). Nevertheless, in the most cases, *P. cinnamomi* has an asexual sporulation, through development of multinucleate sporangia (Hardham 2005) (Figure 5). When conditions favour growth prevails (high soil moisture, soil temperature superior to 10°C) the resting spores germinate and somatic hyphae form multinucleate sporangia that cleave and release motile, biflagellate and wall-less zoospores into the soil water (Figure 5). These zoospores are chemotactically attracted by young fine root exudates, at the contact moment the zoospores encyst, forming walled cysts that germinate and penetrate the tissue. *P. cinnamomi* is able to grow inter- and intracellular showing typical coralloid to irregular and non-septate hyphae. Within 2-3 days in a susceptible host, sporangia will form on the plant surface. The asexual cycle may be repeated million of times in quick succession, rapidly amplifying the inoculum potential in the infected area (Hardham et al. 1994; Erwin and Ribeiro. 1996; Hardham 2005; Jung et al. 2013; Oßwald et al. 2014).

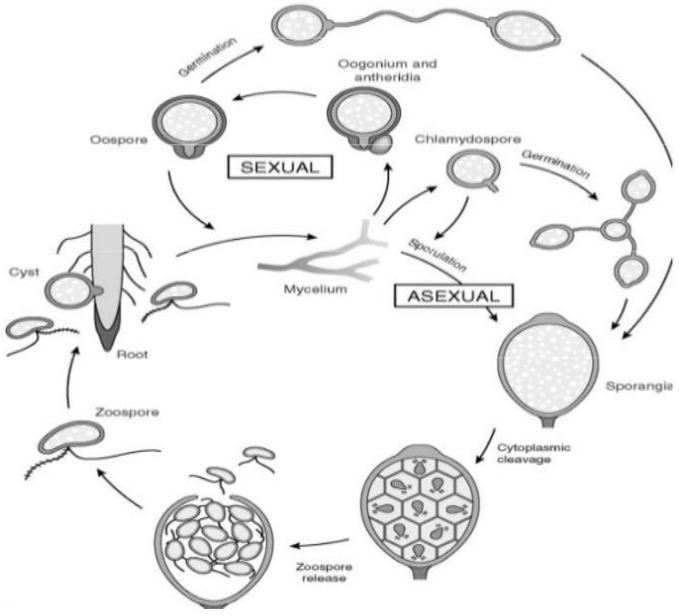

Figure 5. Life cycle of soilborne *Phytophthora cinnamomi* (adapted from Hardham et al. 2005). Sexual and asexual sporulation are shown.

On a local scale, the pathogen can be moved naturally by soil-splash, by wind-blown soil or debris, or by water movement and run-off in drainage/irrigation ditches. The most likely source of more distant movement is in contaminated soil or plant debris. Propagules can also be carried on machinery used for cultivation/harvesting (Hardham 2005; Robin et al. 2012). Cultural control measures include reliving of high soil moisture levels and improving aeration by increasing drainage, and attention to mineral nutrition.

Chestnut blight

Cryphonectria parasitica, a filamentous ascomycete fungus, is a necrotrophic pathogen that incites the chestnut blight disease. The destruction of the American chestnut by *C. parasitica*, was the greatest disaster in the history of forest pathology. It is thought to have been imported on seedlings from Asia and it was first discovered in 1904, on infected American chestnut trees at the Bronx Zoological Park in New York (Anagnostakis, 1987; Anagnostakis, 2001). By 1950, the disease had spread throughout its natural range, and by 1960 had killed an estimated 4 billion trees. In Europe, *C. parasitica* was first recorded in 1938 in Italy and was rapidly spread to the surrounding countries. Chestnut blight became one of the major pathogens that attacked chestnut trees and constituted a serious damage to European chestnut (Anagnostakis 1987; Robin and Heiniger 2001; Jacobs et al. 2015). Currently, *C. parasitica* is distributed along Europe, United States, west Asian and Australia (Figure 6).

The pathogen infects primarily through wounds on stem tissues and kills the above ground portions of trees by girdling the cambium. Once established as germinating conidia (single-celled spores, produced asexually) or ascospores, the fungus grows rapidly through the bark and colonizes the cambial zone. Resistant reactions slow this growth, maintaining the fungus in a superficial canker, whereas susceptible reactions continue development

unimpeded, encircling the stem and causing vascular dysfunction, resulting in death of distal tissues and stem dieback (Anagnostakis, 2012).

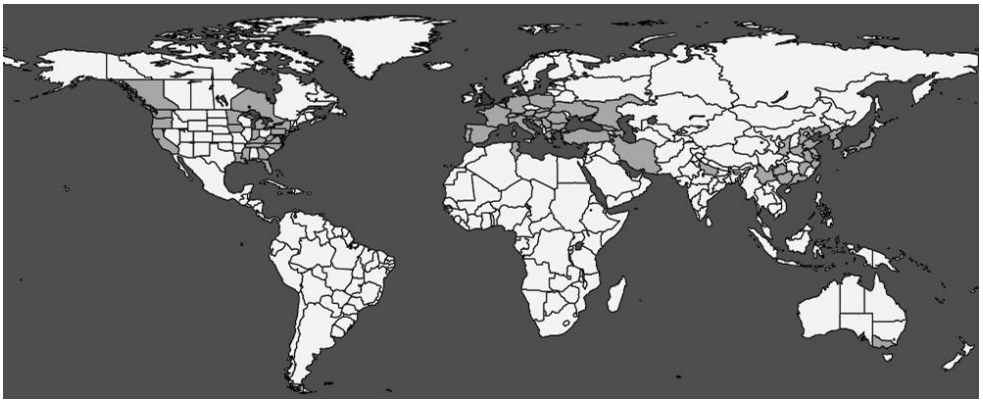

Figure 6. *Cryphonectria parasitica* distribution worldwide (in grey), adapted from European and Mediterranean Plant Protection Organization, Global Database (2016).

Major pests affecting chestnut

More than 50 species of insects are known to damage chestnut, including the *Dryocosmus kuriphilus* (Yasumatsu), which is the most severe insect pest worldwide affecting chestnut. *D. kuriphilus* attacks the vegetative buds of chestnuts and forms a gall, disrupting twig growth and reducing fruiting. Severe infestations may result in the decline and death of chestnut trees. This insect is endemic in China, and was accidentally introduced into Japan (1941), Korea (1963), and the USA (1974) (Abe et al. 2007). In 2002, gall wasp was reported for the first time in Europe in northwest Italy (Brussino et al. 2002). From then, chestnut gall wasp has been spread throughout Europe, being present in many countries (European and Mediterranean Plant Protection Organization, Global Database). Beyond European chestnut, *D. kuriphilus* attacks Asian chestnut species, the American chestnut and their hybrids.

Other important pests affecting chestnut production are: the moth larvae *Cydia splendana* and the weevil *Curculio elephas*. Cydia penetrate the nut through the bur as neonate larvae, and *Curculio* females oviposit through the bur.

Approaches to control diseases and pests

Management of the ink disease and chestnut blight is very difficult since their spores are easily dispersed by water or air, respectively. Nevertheless, sanitary precautions should be applied to introduced plant material, farm machinery and soil, in order to prevent disease establishment or spread.

Biological control

No biological control is known against *P. cinnamomi*. However, the mycorrhization of chestnut roots has been reported as a partial benefic protection to ink disease (Branzanti et al. 1999; Rodrigues and Martins 2006; Martins 2008; Carvalho 2014).

Chestnut blight is one of the few tree diseases in Europe for which biological control is possible, by a phenomenon called hypovirulence. *C. parasitica* strains are infected by a mycovirus (family Hypoviridae) that reduces its virulence and sporulation capacity, creating a superficial or 'healing' cankers that are not lethal for the tree (Griffin, 2000; Anagnostakis, 2001; Robin and Heiniger, 2001; Prospero and Rigling, 2016). In many areas of Europe, hypovirulence has effectively controlled blight spread. However, in most North American stands where biocontrol has been tried, viruses fail to spread among trees and cankers within a tree, severely limiting the use of mycoviruses as biocontrol agents (Jacobs et al. 2015).

D. kuriphilus was successfully controlled in Japan by introducing a parasitoid, *Torymus sinensis* Kamijo (Hymenoptera: *Torymidae*), from China's mainland. Recently, *T. sinensis* was introduced in Europe and may be a

promising method for reducing the pressure of the pest in chestnut forests and orchards (Quacchia et al. 2008).

Chemical

Phosphites (H_2PO_3) are alkali metal salts of phosphoric acid [$HPO(OH)_2$] that show an effective control agent for a number of crop diseases caused by several *Phytophthora* species and therefore, are being promoted and used as chemical control against *P. cinnamomi* (McDonald et al. 2001; Gentile et al. 2009). However, high levels of phosphite in the soil could have a negative environmental impact and also could create an unbalance of the availability of other nutrients (Carvalho 2014).

Genetic modification

Chestnut genetic transformation is possible today because of decades of research on *in vitro* tissue culture, namely somatic embryogenesis and tissue regeneration of both European and American chestnut (Vieitez and Vieitez 1980; Vieitez et al. 1985; Merkle and Wiecko 1991; Sánchez and Vieitez 1991; Carraway and Merkle 1997; Corredoira et al. 2003; Miranda-Fontaiña and Fernández-López 2005; Corredoira et al. 2006).

The first report of successful genetic transformation of European chestnut mediated by *Agrobacterium tumefaciens* using hypocotyl segments from *in vitro*-germinated seedlings and stem segments was published in 1998 by Seabra and Pais (Seabra and Pais 1998). Afterwards, an efficient genetic transformation protocol for *C. sativa* somatic embryos was described (Corredoira et al. 2004). This transformation protocol has been improved by studying the effect of both the genotype and the type of initial explant, in order to increase the tolerance of European chestnut to the diseases (Corredoira et al. 2006; Corredoira et al. 2007; Corredoira et al. 2012). Meanwhile, some partial gene encoding proteins described as pathogenesis-related were isolated and cloned from *P. cinnamomi* inoculated resistant chestnut plants

and also from chestnut cDNA libraries: a cystatin, a beta 1,3 glucanase isoform, an allene oxide cyclase, and a thaumatin-like protein. Chestnut and tobacco explants were transformed by particle bombardment or *A. tumefaciens*, to study the overexpression effect of the isolated genes on plant resistance to *P. cinnamomi* (Serrazina 2004; Santos 2010).

The first reports on transgenic American chestnut tissues were carried out in 1994, using biolistics to transform pro-embryogenic masses derived from immature zygotic embryos (Carraway et al. 1994). More recently, a candidate gene for *C. parasitica* resistance, oxalate oxidase gene (OxO), was introduced (*A. tumefaciens*-mediated) in *C. dentata* somatic embryos (Polin et al. 2006). The process has been improved in order to increase transformation and regeneration rates and transgenic plants are already in the field (Rothrock et al. 2007; Andrade et al. 2009; Zhang et al. 2013). Crossing blight resistant transgenic plants with several genotypes of American chestnut will produce populations with great genetic variability, suitable for the American chestnut reforestation.

Chestnut breeding

Climate change is predicted to enhance the intensity and distribution of chestnut diseases in the near future (Conedera et al. 2011; Thompson et al. 2014). Consequently, new plantations with improved genotypes are increasingly demanded for the preservation or restoration of chestnut species, even in contaminated areas. Chestnut breeding programs have been designed aiming to develop genetically improved genotypes in an economically efficient manner.

Asian species show great resistance to ink disease and chestnut blight (Crandall et al. 1945) and therefore, they have been used as donors of resistance for the breeding programs, through selection and hybridization. Japanese chestnut is one of the most important sources of resistance to *P.*

cinnamomi while Chinese chestnut is considered the most resistant *Castanea* species to *C. parasitica*.

At the beginning of the twentieth century, European researchers from Portugal, Spain, France and Italy introduced seedlings of *C. crenata* and *C. mollissima* into Europe (Elorrieta 1949). These species were resistant to ink disease, but their nuts and timber were not appreciated by farmers and consumers, mainly because of poor vigor and poor quality and peeling, respectively. Moreover, they presented difficulty to adapt to some European climatic characteristics, such as early spring frost and summer drought (Elorrieta 1949). When used as rootstocks, the incompatibility with local varieties was common.

Later, interspecific hybrids were produced in Portugal, Spain and France and some resistant rootstocks were obtained successfully, combining resistance to *P. cinnamomi*, easy propagation/cultivation and good compatibility for grafting. Therefore, hybridization has been the main breeding method used till now to introgress resistance to ink disease into European chestnut, as well as, to blight into American chestnut, and gall gasp into Japanese and European chestnut (Costa et al. 2011; Pereira-Lorenzo et al. 2012; Hebard et al. 2014b; Fernández-Cruz 2015). Hybridization was also established in Japan to introgress the easy peeling trait into Japanese cultivars (Takada et al. 2012; Nishio et al. 2013).

In France and Spain, chestnut breeding programs produced some *P. cinnamomi* resistant hybrid rootstocks, which have been commercialized as rootstocks. Their *P. cinnamomi* resistance varies from low/medium to very high and the compatibility with local cultivars is excellent. They are propagated by stooling, cuttings, or *in vitro* culture and have been broadly used (Breisch 1995; Pereira-Lorenzo 1997; Pereira-Lorenzo et al. 2001). French popular rootstocks include: CA 07 ‘Marsol’ (moderately resistant); CA 74 ‘Maraval’ (resistant, low vigor); CA 118 ‘Marlhac’ (moderately resistant,

but able to grow at temperatures less than -10°C); CA 90 'Ferosacre' (resistant, but sensitive to temperatures less than -10°C).

In Portugal, interspecific hybridizations were initiated in the fifty's, by Bernardino Gomes, Columbano Fernandes and Vieira Natividade (Natividade 1947; Guerreiro 1948; Fernandes 1955; Guerreiro 1957). Resistant genotypes were produced, but unfortunately the genetic resources obtained were not preserved properly and little information is available about that research. Recently, a new Portuguese chestnut breeding program was established for introgression of ink disease resistance into European chestnut, in which the present PhD project was integrated (Costa et al. 2011). In 2006, controlled crosses between European and Japanese chestnut were initiated, and so far, 155 progenies were obtained. Mapping and transcriptomic approaches have been integrated in order to unveil the chestnut resistance mechanisms to *P. cinnamomi* infection. The major outcomes resulting from this breeding program are presented in this thesis.

In the USA, in the early 1980s, The American Chestnut Foundation (TACF) has developed a backcross breeding program using blight resistant Chinese species as the donor and American chestnuts as the recurrent parent was proposed to produce blight resistant American chestnut (Diskin et al. 2006; Hebard et al. 2014b). The specific steps include: 1) perform three backcross generations (BC_3) with selection for resistance (after blight exposure) at each generation to ensure retention of Asian resistance genes; 2) inter-crossing the selected BC_3 trees to produce BC_3F_2 populations which fully segregate for resistance; 3) select high resistant individuals in the BC_3F_2 populations (93% of seedlings should have morphological characteristics of American chestnut with 100% Chinese resistance to blight); 4) establish the selections in seed orchards to produce planting stock for forest planting (Diskin et al. 2006). Decades of research are needed to obtain the ideal plant material. During this period, extensive genetic and genomic resources have been developed as tools for assisting the traditional methods. Genomic resources

include molecular markers, high-density genetic and physical maps and QTLs for blight resistance (more details in 'Genomic resources' section).

Castanea - *Phytophthora cinnamomi* molecular interactions

Many studies have evaluated physiological, biochemical (Fleischmann et al. 2004; Fleischmann et al. 2005; Portz et al. 2011), histological (Phillips et al. 1987; Portz et al. 2011; Li et al. 2014; Ruiz Gómez et al. 2014; Redondo et al. 2015) and molecular (Coelho et al. 2011; Moy et al. 2007; Restrepo et al. 2005; Schlink, 2010; Serrazina et al. 2015) interactions between *Phytophthora spp.* and their hosts, mainly susceptible ones.

As *P. cinnamomi* is a broad host range pathogen, it has evolved sophisticated mechanisms to manipulate plant cells increasing their vulnerability. *P. cinnamomi* is a hemibiotrophic pathogen with an initial biotrophic stage during early infection followed by necrotrophic colonization of the host tissue (Latijnhouwers et al. 2003). Understanding molecular and physiological interactions between *P. cinnamomi* and their host plants is an important step for the development of disease control strategies.

Some crops and model plants have been used to study interactions between plant and pathogens (including some *Phytophthora* species), increasing the knowledge about plant immune system (Huitema et al. 2004; Moy et al. 2007; Attard et al. 2008; Eshraghi et al. 2014). Plants respond to pathogen infection using two innate immune systems: PAMPs (pathogen-associated molecular patterns)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl 2006). Both plant immune responses aim at interfering with pathogen introgression and spread. PTI constitutes the basal defense response that often successfully inhibits disease development. However, depending on pathogen virulence, environmental conditions and host susceptibility, pathogens are able to secrete numerous effector proteins (Schornack et al. 2009). Effectors interact with plant cell especialized receptors at the cell wall, plasma membrane or in the cytoplasm thereby

reprogramming the host cell to accommodate the needs of the pathogen. Intracellular disease resistance proteins mediate recognition of effectors entering the host cell and elicit effector-triggered immunity (ETI) (Jones and Dangl 2006; Stael et al. 2015). Besides local immune responses, PTI and ETI activate long-distance defense reactions, such as systemic acquired resistance (SAR) (Durrant and Dong 2004). Plants also have the ability to defend themselves against different pathogens also by regulating transcriptional activity, induction of tailored defense responses including callose deposition, cell wall thickening and production of reactive oxygen species (ROS) (Jones and Dangl 2006; Stael et al. 2015; Herrera-Vásquez et al. 2015).

Plant hormone balance also play a key role in determining the outcome of plant–pathogen interactions. The best characterized defense hormones include salicylic acid (SA), jasmonic acid (JA), absisic acid (ABA) and ethylene (Spoel and Dong 2008; Bari and Jones 2009; Pieterse et al. 2009). If all defense responses fail to inhibit pathogen ingress, the plant cell under attack can undergo hypersensitive cell death (Kamoun et al. 1999; Mur et al. 2008; Choupina et al. 2014). Nevertheless, *Phytophthora* species have evolved a range of counter-defense mechanisms that can inhibit all host defense processes mentioned (Hardham and Blackman 2010). Despite all the knowledge acquired, molecular mechanisms involved in woody plants resistance to *Phytophthora* species are poorly understood. This research is challenging because no genome data is available for many wood species and so, identification and characterization of *Phytophthora* resistance genes is necessary. Nevertheless, strategies of attack and defense in plants-oomycete interactions were recently reviewed (Oßwald et al. 2014; Fawke et al. 2015). Apart from the *Castanea* genus, the most characterized interactions in Fagaceae family are *Quercus suber* - *P. cinnamomi* (Coelho et al. 2006; Coelho et al. 2011; Ebadzad and Cravador 2014) and *Fagus sylvatica* - *P. citricola* (Portz et al. 2011; Schlink, 2010). *Q. suber* - *P.*

cinnamomi interactions have been studied by cloning and characterization of a set of candidate resistance genes (Coelho et al. 2006; Coelho et al. 2011; Ebadzad and Cravador 2014). Moreover, a hypothetical mechanisms model was proposed for five of those genes for which expression was increased 24hpi (Coelho et al. 2011). Molecular interactions observed between *F. sylvatica* and *P. citricola* was characterized by transcriptional changes after infection. Results indicated that *P. citricola* escapes the main recognition systems and/or suppresses the host's response (Schlink 2010). As a first step to identify transcripts involved in the *Castanea* - *P. cinnamomi* interaction, our research group identified and characterized root transcriptomes expressed sequence tags (ESTs) differentially expressed in European chestnut (*Castanea sativa*) and Japanese chestnut (*Castanea crenata*), in response to inoculation with *P. cinnamomi* (Serrazina et al. 2015, Chapter III). Nevertheless, the pathogenic process should comprise a network of molecular signaling and interaction events in different time points after *P. cinnamomi* infection that were not yet achieved in *Castanea* spp.

Research objectives and thesis layout

The general aim of the work here described was to provide new insights about the *Castanea* resistance mechanisms to *P. cinnamomi* infection using different approaches: genomics, phenomics and transcriptomics.

The outcomes of this project constitute an essential contribution to the understanding of chestnut response to *P. cinnamomi* based on an elite plant material created from the breeding program, that segregates for the trait of resistance. The development of improved chestnut genotypes with increased resistance to pathogens and the production of genomic resources for future molecular assisted selection will also constitute an asset for the improvement and adaptation of woody plants, mainly belonging to Fagaceae family, to biotic stresses.

The specific objectives of this work were:

1. Perform new controlled crosses between *C. sativa* and *C. crenata* in order to increase the hybrid chestnut population obtained in 2006 and 2009;
2. Phenotype *C. sativa* x *C. crenata* (SC) hybrid progenies obtained previously and obtained from new crosses. Phenotype a small population from *C. sativa* x *C. mollissima* (SM) controlled crosses to compare levels of resistance among progenies with different donors of resistance;
3. Construct the first interspecific genetic map for *C. sativa* x *C. crenata* population through genotyping of parents and progenies with molecular markers: microsatellites or Simple Sequence Repeats - SSRs and Single Nucleotide Polymorphism - SNPs;
4. Perform DNA marker:trait association analysis to identify genomic regions that explains the phenotypic variation in the SC population, by identification of Quantitative Trait Loci.
5. Identify candidate genes related with the resistance to *P. cinnamomi* by comparing the root transcript profiles of resistant and susceptible species, before and after inoculation;
6. Evaluate the expression of genes potentially involved in the resistance to *P. cinnamomi* in parental genotypes (*C. sativa* and a *C. crenata*), as well as, in hybrid genotypes with different responses to *P. cinnamomi*.
7. Localize in the genetic map the differential expressed genes by developing molecular markers on sequences obtained from the root transcriptomes.

This thesis presents all the work organized in scientific articles, from Chapter II to V, and the work followed the steps described in Figure 7. Final conclusions and future perspectives are discussed in Chapter VI.

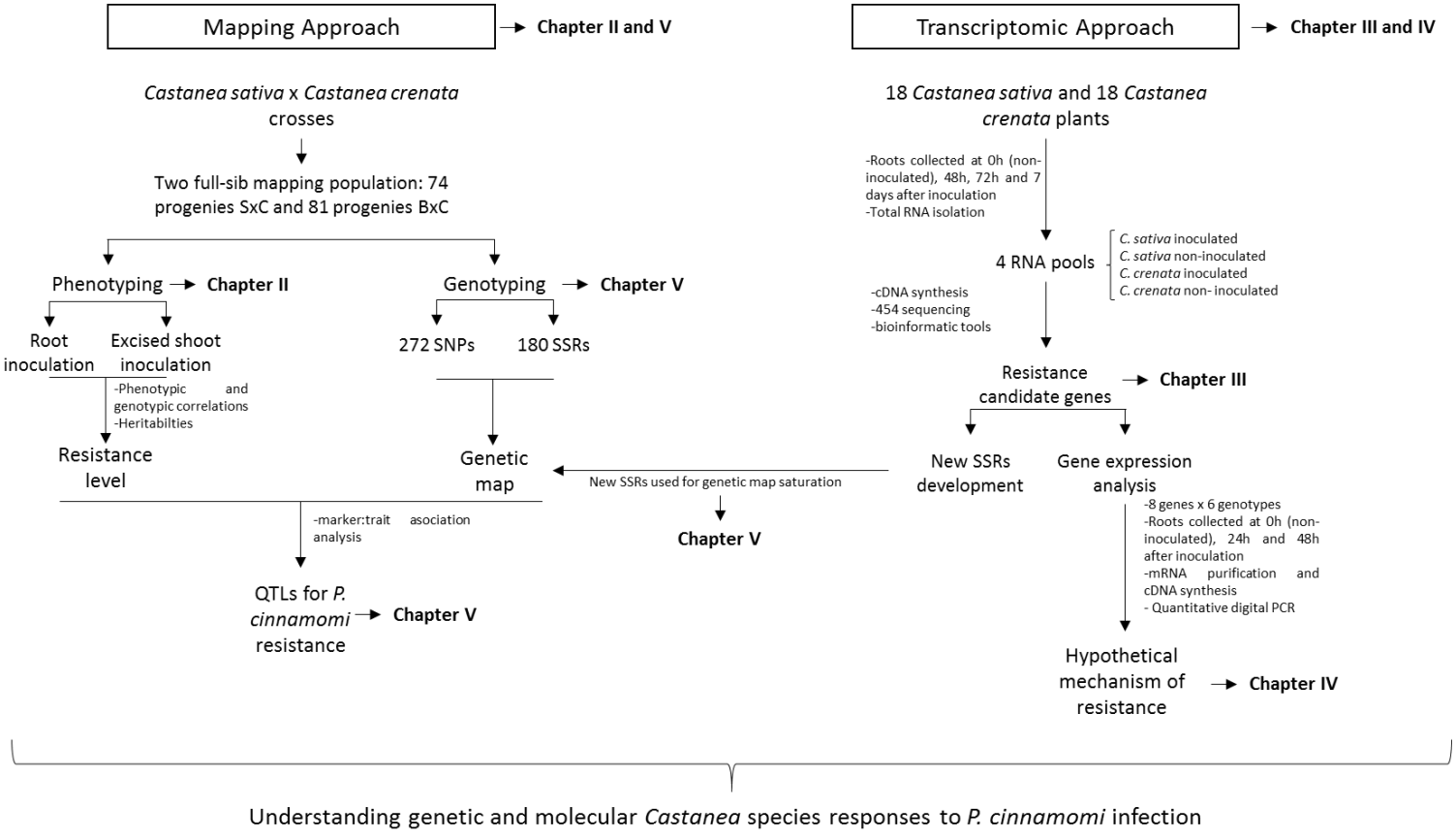

Figure 7. General organization of the research and thesis, highlighting the main approaches and techniques used during thesis studies.

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Chapter II

Phenotyping *Castanea* hybrids for *Phytophthora cinnamomi* resistance

Part of the work presented in this chapter was published in the following research publication:

Santos C., Machado H., Correia I., Gomes F., Gomes-Laranjo J. and Costa R. (2015) Phenotyping *Castanea* hybrids for *Phytophthora cinnamomi* resistance. *Plant Pathol.* 64, 901–910. doi: 10.1111/ppa.12313

In this research paper Carmen Santos participated in the experimental design, laboratory experiments, results interpretation and paper writing.

Abstract

Castanea sativa is susceptible to *Phytophthora spp*., a serious root pathogen causing ink disease, while *C. crenata* and *C. mollissima* show resistance to infection. Interspecific controlled crosses were produced to introgress resistance genes from the resistant species into the susceptible *C. sativa*, and three mapping populations were created. *Phytophthora cinnamomi* resistance of the progenies *C. sativa* x *C. crenata* and *C. sativa* x *C. molissima* were evaluated by root and/or excised shoot inoculation tests. The number of days of survival after root inoculation was the best discriminator of resistance to *P. cinnamomi* while the percentage of shoots with internal lesions was the symptom most associated with survival. The lesion progression rate in the excised shoot inoculation test was strongly and negatively correlated with survival in the root inoculation test. The excised shoot inoculation test appears to be a reliable approach for screening the resistance of chestnut genotypes to *P. cinnamomi*. Therefore, a recently obtained progeny (in 2015) was phenotyped using the excised shoot inoculation test. Strong genetic correlations were obtained between survival and ink disease symptoms and among symptoms, indicating that common or linked genes might influence resistance to *P. cinnamomi*. The most resistant genotypes selected from this study will be tested for other commercial variables, such as ease of vegetative propagation and stock–scion compatibility.

Keywords: *Castanea* hybrids, heritability, phenotypic and genetic correlations, *Phytophthora cinnamomi*

Introduction

The genus *Castanea* belongs to Fagaceae, a plant family that dominates much of the climax hardwood forests of the Northern Hemisphere (Manos et

al. 2008). The European chestnut (*Castanea sativa*) is considered to be the only native species in Europe. Chestnuts are multipurpose trees being used in the food industry, for its edible nuts, in the wood industry, as timber and also for ecological and landscaping purposes, having a major economic importance in the Mediterranean region.

Chestnut fruit production has declined considerably in southwestern Europe due to social changes and cultural development, and particularly to the emergence of heavily damaging diseases. Ink disease, caused by *Phytophthora* spp. is one of the most destructive diseases affecting *Castanea sativa*. *Phytophthora cinnamomi* is an aggressive root pathogen, originally from the southeast Asian tropics (Hardham 2005). Nowadays, *P. cinnamomi* is widespread and continues to be destructive in forests of Mediterranean countries, Australia, southeast USA, southern California and more recently it was recognized as a danger to forests in western North America (Robin et al. 2012).

Phytophthora cinnamomi has an exceptionally wide host range, being able to invade more than 3 000 plant species around the world (Hardham 2005; Cahill et al. 2008). Currently, it is the most important *Phytophthora* pathogen of forest trees; besides chestnut, *P. cinnamomi* causes root diseases in eucalyptus, oaks, pines and members of the *Ericaceae* family, as well as, several agricultural crops (Robin et al. 2012).

Disease symptoms in chestnut are similar to other species: ink disease causes root rot, with necrosis of tap root, which extends to the lateral roots and the collar. *P. cinnamomi* infection induces necrosis of the cambial and xylem tissues, causing interference with transpiration from roots to shoots, and consequently causes wilting of leaves and dieback of young shoots (Marçais and Dupuis 1996; Robin et al. 2001; Vannini and Vettraino 2001; Hardham 2005; Gomes-Laranjo et al. 2009).

The pathogen spreads slowly through root-to-root contact and more rapidly in presence of water. Human activities that move soil and the planting of

infested nursery stock intensify pathogen spread (Robin et al. 2012). With changing climates, *P. cinnamomi* is expected to expand its area of destruction, mainly in Europe and North America (Robin et al. 2012; Thompson et al. 2014).

In Portugal, ink disease has become widespread, since *P. cinnamomi* was first recorded, in 1838. Despite the protection measures taken, it is still a great threat to chestnut orchards, as fruit and timber production is negatively impacted. Chestnut production is an important source of income for rural populations and so new plantings have been carried out while old orchards are being restored. In Europe, chestnut breeding for ink resistance began with the introduction of the Asian chestnut germplasm, which is resistant to the main diseases: ink and blight (caused by *Cryphonectria parasitica*). Japanese and Chinese species (*Castanea crenata* and *Castanea mollissima*, respectively) were introduced since 1917 in several southern European countries (Elorrieta, 1949). However, the low value of the Asian species as timber and fruit producers was notable. They also presented low compatibility for grafting with local sweet chestnut varieties (Elorrieta, 1949). In Portugal, the first interspecific hybridizations were initiated in 1948 by Bernardino Barros Gomes to introduce resistance to ink disease in *C. sativa* (Guerreiro, 1948; Guerreiro, 1957). The objectives of these programs were to breed for resistance to ink disease, as well as to produce rootstock or varieties selected for early nut production or better wood production as compared with Asian species (Fernández-López 2011).

In 2006, interspecific controlled crosses were performed between *C. sativa* and *C. crenata* (SC) and between *C. sativa* and *C. mollissima* (SM) in order to introgress the resistance from Asian species into the European (Costa et al. 2011). The main goal of our ongoing program was to produce a hybrid segregant population to perform DNA marker-phenotype association analysis to identify genomic regions related with the ink disease resistance (Quantitative Trait Loci, QTL). For this purpose, it is crucial to determine

accurately both genotype and phenotype of each hybrid progeny. However, there are some limitations for the determination of the resistance of *Castanea* spp. to *P. cinnamomi*.

Different methods have been used used for screening the resistance of chestnut to *Phytophthora* spp. by different authors.: i) root inoculation using seedlings (Vettraino et al. 2001; Santini et al. 2003; Robin et al. 2006; Jeffers et al. 2009); ii) root inoculation using cuttings (Miranda-Fontaiña et al. 2007) or iii) plantlets from micropropagation (Cuenca et al. 2009) and iv) direct inoculation on the top of excised or intact stem/shoot from seedlings or clones selected in the field (Guedes-Lafargue & Salesses 1999; Fernández-López et al. 2001; Vettraino et al. 2001b; Robin et al. 2006; Miranda-Fontaíña et al. 2007; Cuenca et al. 2009). There are advantages and drawbacks to each method; in particular, shoot inoculation is easy to achieve and enables the screening of a high number of individuals at low cost (Fernández-López 2011), but a criticism of this method is that *P. cinnamomi* is a root pathogen. The objectives of the present study were (i) to select the best resistance discriminators from root and excised shoots inoculation tests and clarify their correlations; and (ii) to assess the resistance to *P. cinnamomi* and evaluate its heritability in progenies of three *Castanea* sp. segreganting populations.

Material and Methods

Plant material

Three full-sib progenies were obtained from artificial controlled crosses: *C. sativa* (cultivar Aveleira) x *C. crenata2* (SC), *C. sativa* (cultivar Aveleira) x *C. mollissima* (SM) and *C. sativa* (cultivar Bária) x *C. crenata1* (BC) in 2006, 2009, 2012 and 2015. *C. sativa* female flowers were isolated before pollination season by placing pollination bags on the branches and cutting off the closer catkins. The parental line of the *C. sativa* (cultivar Aveleira) was the same for crosses with *C. crenata2* and *C. mollissima*. Catkins from *C. crenata* and *C. mollissima* were collected and dried the day before

pollination. On the day of pollination, pollen was removed from catkins and filtered. Then, pollen was placed on the stigmas using a paintbrush or a piece of glass (only the pollen grains are adhered to the glass). Pollinated flowers were covered with paper or polyester pollination bags or and kept until the end of pollination season. Subsequenlty, pollination bags were replaced by net bags in order to collect the seeds. The crosses were performed at the germplasm bank of Universidade de Trás-os-Montes e Alto Douro, Vila Real for SC and SM crosses, and in a private orchard in Marvão for BC crosses, both in Portugal.

A total of 142 F1 genotypes were tested for *P. cinnamomi* infection by either root inoculation test or excised shoot inoculation tests or by both (Table 1). For root inoculation test, 137 plantlets were produced from 20 genotypes by *in vitro* propagation from buds of mother plants. At the time of inoculation, plantlets were different ages, as determined by the number of days after acclimatization, but were most frequently 80 days old; aerial parts were 16.66 cm on average.

Table 1. Number of individuals from *Castanea sativa* (cultivar Aveleira) x *C. crenata2* (SC), *C. sativa* (cultivar Aveleira) x *C. mollissima* (SM) and *C. sativa* (cultivar Bária) *x C. crenata1* (BC) crosses tested by root inoculation and excised shoot inoculation.

Test method	SC	SM	BC	Total
Root inoculation	16	4	0	20
Excised shoot inoculation (total)	45	18	76	139
Excised shoot inoculation (spring)	30	17	0	47
Excised shoot inoculation (autumn)	42	18	76	136
Total	48	18	76	142

The excised shoot inoculation tests were carried out in spring and autumn of 2012 for a total of 63 SC and SM progenies. For BC progenies, the excised shoot inoculation tests was performed in autumn of 2016 (Table 1). A total number of 1034 shoots were collected from the mother plants. Both

experiments were performed in a controlled chamber with temperatures ranging between 18 and 22ºC, photoperiod 16h light/8h dark and 65% of relative humidity.

Inoculum of *P. cinnamomi*

In all experiments, the same isolate of *P. cinnamomi* was used (IMI 340340), which was selected, as the most virulent, following tests using several isolates (Abreu et al. 1999). The high pathogenicity of this isolate in European chestnuts was also confirmed by Dinis et al. (2011).

For the root inoculation test, the *P. cinnamomi* inoculum was prepared by growing mycelia on sterilized millet seeds (*Ponicium mileaceum*), which were thoroughly moistened with V8 medium broth [20% (v/v) with 3 g/L of $CaCO_3$]. Afterwards, this mixture was incubated for 3 weeks in darkness at 24°C.

For the excised shoot inoculation test, *P. cinnamomi* was grown on potato dextrose agar for 6 days in darkness at 22ºC.

Root inoculation test

Four experiments of root inoculation were carried out using clonal plantlets placed in sterile substrate. For each experiment, one or two plantlets of each genotype, were used as a control, without inoculation. For root inoculation, *P. cinnamomi*-infected millet seed inoculum was carefully placed into the substrate (600 mL) of each pot, at a concentration of 5% (v/v). Mostly, eight replicates per genotype were inoculated, but, due to limitations of *in vitro* propagation, this was not alwalys possible. Therefore, the mean number of plants per genotype was 6.85.

Inoculated plants and controls were placed separately in different trays and each pot was flooded for 1h, three times a week, to stimulate zoospore release and to promote disease development.

P. cinnamomi was recovered from water collected from flooding process, using a modified baiting technique adapted from Jung et al. (1996).

The experimental design was adapted from Miranda-Fontaíña et al. (2007). For each individual, the days of survival after inoculation were recorded, until 100 days after inoculation (dai). After death, plantlets were removed from the soil and the roots were gently washed to observe and record ink disease symptoms.The level of root rot was assessed on a scale from 1 to 6, according to Miranda-Fontaíña et al. (2007), where 1 indicates the least severe level of root rot and 6 indicates the most severe level. The percentage was then used to rate the level of root collar rot on a scale of 1-6 (1, no rot; 2, 0.1-9.9% rot; 3, 10-19.9% rot; 4, 20-29.9% rot; 5, 30-49.9% rot and 6, >50% rot). Shoot internal and external lesions were recorded as the percentage length of internal and external lesion of the longest shoot, respectively. Biomass parameters were also evaluated for each plantlet: leaf and shoot dry weight (g) and root dry weight (g). In order to determine the dry weight, leaves and shoots were separated from roots and both parts were dried at 60ºC for 2 days.

At the end of each experiment, plantlets that did not die during the test were analysed without destruction and were transplanted to new pots that were placed in a greenhouse with controlled conditions (20-25ºC). In the spring of the next year, the number of plantlets that showed budburst was recorded.

Excised shoots inoculation test

Excised shoot inoculation test for SC and SM populations took place in spring, using 47 genotypes (eight genotypes were common to the root inoculation test), and in autumn, using 60 genotypes (16 common to the root inoculation test). The 76 progenies from BC cross were screnned in autumn, 2015 (Table 1). Excised shoots, the majority with a length of 15 cm, were collected from each mother plant. The mean number of excised shoots inoculated per genotype was 7.94 in spring and 4.86 in autumn. All apart from two upper leaves were removed to reduce evapotranspiration. The diameter of the top of each excised shoot was recorded before inoculation.

Mycelial plugs of *P. cinnamomi* were then placed on the top of the shoots and were covered with an aluminium sheet to avoid desiccation. Replicates were distributed randomly in three trays with perlite and water, in an environmental controlled chamber. Five days after inoculation, the aluminum sheets were removed from each shoot, when colonization by the pathogen had occurred. Resistance to *P. cinnamomi* was evaluated by measuring the visible external lesion length (LL) at 5, 7, 9, 12 and 14 dai. The lesion progression rate (cm/day) was calculated for each genotype, using the following formula:

$$\frac{\left(\frac{LL_{5\,dai}}{5}\right)+\left(\frac{LL_{7\,dai}-LL_{5\,dai}}{2}\right)+\left(\frac{LL_{9\,dai}-LL_{7\,dai}}{2}\right)+\left(\frac{LL_{12\,dai}-LL_{9\,dai}}{3}\right)+\left(\frac{LL_{14\,dai}-LL_{12\,dai}}{2}\right)}{5}$$

Statistical analysis

Analysis of variance (ANOVA) was conducted for root and shoot variables using linear mixed effects models of the general form $y = X\beta + Z\gamma + \varepsilon$, where y is the vector of observations; X and Z are design matrices of the parameters associated to fixed and random effects, respectively; β and γ are vectors of fixed effects (including the general mean) and random effects, respectively; and ε is the vector of residual errors. In preliminary data analyses, resistance from SC and SM crosses was evaluated separately for each cross by specifying a two-level 'Family' effect (SC and SM). Because no significant differences were found between the two full-sib families, the family effect was dropped from the model. For the root inoculation test data, Age, Genotype, Inoculation, Inoculation Date and Genotype x Inoculation Date were fitted as fixed effects (β vector); for the excised shoot inoculation test data, Length, Genotype, Season and Genotype x Season were treated as fixed effects. *F*-tests were used to test the significance of the fixed effects and of genotype mean comparisons, the latter implementing the Tukey adjustment and a matching letter display (adapted from Piepho, 2012). If significant, the

covariate age was included in multiple comparison of means and in least squares means (LSM) estimation (at Age=80 days; not for level of root rot and level of root collar rot); and similarly for the covariate Length (at Length=15cm). To gain insight into the variation pattern, a further partition of *F*-tests was performed on the shoots test data for SC and SM populations, to search for the season and day within season where differences between genotypes were maximized. The Restricted Error Maximum Likelihood (REML) estimation was used, with a significance level of α=0.05 and the Satterthwaite approximation for degrees of freedom (d.f.) when data were missing. Plots of the Studentized residuals showed conformation to the assumptions of the mixed model, except for survival, level of root rot, level of root collar rot and lesion length, which were modelled with a log-normal distribution; shoot internal lesion and shoot external lesion, which were arcsine-transformed and modelled with a Gaussian distribution, for all statistical tests (LSM are reported on the original variable scale). Whenever appropriate, variance heterogeneity between inoculation dates or seasons (root and excised shoot inoculation test, respectively) was fitted by adjusting different residuals by group ($\boldsymbol{\varepsilon}$ vector).

Phenotypic and genetic correlations were estimated for different variables measured in the same plants of the root inoculation test (based on all observations); and between pairs of variables from the two tests (based on genotype LSM). Phenotypic correlations were analysed with the nonparametric (distribution-free) Spearman's correlation coefficient. Genetic correlations were obtained from the additive genotypic covariance matrix in multivariate repeated measures analysis using REML estimation. The transformation log (2y + 1) was applied to data, in order to meet the model assumptions.

Genotype-level narrow-sense heritabilities were estimated with the REML approach for root and shoot variables as $h^2 = \frac{\sigma_a^2}{(\sigma_a^2+\sigma_e^2)}$ where additive genetic

variance σ_a^2 was the genotype variance component, implemented with an additive relationship matrix obtained with pedigree information; σ_e^2 was the residual variance component; and $\sigma_a^2 + \sigma_e^2$ was the phenotypic variance. Age (covariate) and Inoculation Date (root inoculation test) and the covariate Length (excised shoot inoculation test) were included as fixed effects in the heritability calculation. Data transformations and distributions used to model the response variables were applied as explained above. Approximate standard errors (SE) of heritabilities were obtained by Taylor series expansion. All analysis were conducted using SAS® v 9.3 software (GLIMMIX, CORR, and INBREED procedures).

Results

Root inoculation test

All control plantlets survived until the end of the experiment. No symptoms associated with *P. cinnamomi* were observed in the roots, collar and shoots of the control plantlets, indicating that cross contamination did not occur. A reduction in the growth of inoculated plantlets was observed, when compared with non-inoculated plantlets. Over the course of the experiment, the average growth of *P. cinnamomi*-inoculated and control plantlets was 1.23 cm and 1.71 cm, respectively.

At the end of experiment, only 18 inoculated plantlets (13.14%) survived, corresponding to seven different genotypes. After death, plantlets showed the typical visual symptoms of *P. cinnamomi*: chlorosis and wilting of leaves and die-back of shoots. Susceptible plantlets showed high levels of root rot, characterized by long necrotic lesions in roots, which in some cases extended to collar and shoots.

Analysis of variance revealed a highly significant effect of age at inoculation on survival and shoot internal lesion (Table 2). Genotype mean values obtained for survival and adjusted for the age covariate effect, showed that there were big differences in the resistance pattern obtained between

different genotypes; the most susceptible genotype (SC918) survived only 10 days, while the resistant ones survived up to 80 dai (Table 3) and the average time of survival was 33.44 ± 3.36 days (Table 2).

Table 2. Effect of the Age, Genotype, Inoculation, Inoculation Date, and Genotype x Inoculation interaction on days of survival and ink disease symptoms for all plantlets root inoculated and controls.

Effect	Days of survival	Level of root rot	Level of root collar rot	Shoot internal lesion	Shoot external lesion
Age[a]	22.06***	0.02	3.63	13,04***	5.92*
Genotype	11.01***	3.63***	2.09*	5,40***	1.54
Inoculation	488.00***	2229.32***	210.98***	126,28***	63.74***
Inoculation date	7.69***	2.87*	3.90*	7,49***	9.11***
Genotype x Inoculation	11.26***	5.21***	2.67**	6,41***	1.24
Mean values ± SE	33.44 ± 3.36	4.80 ± 0.12	4.70 ± 0.24	69.45 ± 6.51	16.73 ± 2.01

[a]Age: days after acclimatization.
F-test significance is indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$. Least squares mean values ± standard-errors (SE) adjusted for the covariate Age for survival, shoot internal lesion and shoot external lesion are represented on the original variable scale (only for inoculated plantlets). Level of root rot and collar root rot was registered using a scale that varies from 1 to 6 and shoot internal and external lesion were calculated, by the percentage of internal and external lesion of the longest shoot, respectively.

Fifty-five percent of the overall genotypes had a survival below average and 20% did not survive more than 15 dai; however 45% of them (nine genotypes) survived beyond 33 days. At the end of experiment the majority of individuals belonging to seven genotypes (SC24, SM904, SC51, SC55, SC09, SC01, SC57) survived after root inoculation and sprouted in the following spring.

Table 3. Ranking of genotypes tested by root inoculation, according to the least squares means (LSM) of days of survival, levels of root and collar rot, and shoot internal and external lesion, on the original variable scale (adjusted for the covariate Age at 80 days).

Genotype	Days of survival		Level of root rot		Level of root collar rot		Shoot internal lesion		Shoot external lesion	
	LSM	Test	LSM	Test	LSM	Test	LSM	Test	LSM	Test
SC01	62.36	ab	4.44	ac	4.64	ac	31.32	cf	23.63	ab
SC09	65.29	ab	4.87	ac	4.74	ac	32.76	ef	15.00	bc
SC19	18.69	def	5.48	a	5.25	a	69.72	cde	15.60	bc
SC24	77.45	abcd	2.74	e	2.08	bc	53.50	bdf	15.50	bc
SC32	18.53	def	5.67	a	6.00	a	100	bd	14.00	bc
SC36	44.51	abce	5.48	acd	4.78	ab	29.95	bdf	23.50	b
SC51	72.29	af	2.69	de	1.93	bc	58.48	bdf	7.71	bc
SC55	70.30	ab	4.07	bc	2.06	b	14.58	f	17.00	bc
SC57	59.22	abc	3.19	cd	3.93	ab	23.85	ef	8.00	c
SC903	12.49	ef	4.86	ac	5.60	a	72.64	abc	12.30	bc
SC904	12.64	f	4.41	ac	3.93	ab	92.09	abe	16.10	bc
SC912	16.68	def	5.44	a	4.68	a	100	bd	12.60	bc
SC914	28.48	cf	5.01	ac	4.34	ab	52.07	adf	19.00	ab
SC916	34.20	bf	5.67	ab	3.02	ab	41.49	bdf	14.40	bc
SC918	10.25	f	4.60	ac	5.12	a	100	bd	9.77	bc
SC919	16.25	def	5.57	a	5.81	a	100	ab	16.70	ab
SM901	25.13	cf	5.23	acd	4.53	ab	45.79	bdf	4.16	bc
SM904	75.70	a	5.01	acd	3.68	ab	41.89	ef	8.25	ac
SM906	14.98	bf	5.15	acd	6.00	ab	100	abc	23.20	bc
SM919	15.04	ef	5.54	a	5.73	a	100	b	20.50	ab

Estimated least squares greater than 100%, due to the model adjustment for the covariate Age at 80 days, were replaced by 100%. Means followed by different letters in the same column are significantly different at $P \leq 0.05$, using the Tukey method; the letter display of the mean comparisons was obtained by adapting the macro described in Piepho (2012) (for the multiple-comparison tests, the normality assumption was approached as described in the statistical analysis section). The LSM of variable for the 7 most resistant genotypes are underline. Level of root rot and collar root rot was registered using a scale that varies from 1 to 6 and shoot internal and external lesion were calculated, by the percentage of internal and external lesion of the longest shoot, respectively. The SC918 genotype showed the highest susceptibility to *P. cinnamomi* whereas SC24 was the most resistant.

The analysis of variance (Table 2) for the effects of Age, Genotype, Inoculation (i.e. inoculation or non-inoculation), Inoculation Date and the interaction between Genotype and Inoculation (G x I) revealed that these effects were significant for most of the variables analysed. For all plantlets tested (with root inoculation and control), there was a highly significant effect

of Age, Genotype, Inoculation, Inoculation Date and G x I on survival and shoot internal lesion. Inoculation had the strongest effect on all variables, as expected. Genotype had a highly significant influence on survival, level of root rot and shoot internal lesion, while level of root collar rot was less influenced by Genotype. On the other hand, the Genotype effect had no significant influence on shoot external lesions (Table 2).

The severity of symptoms decreased progressively from the point of inoculation (roots) to shoots. Depending on the genotype, the root rot was either limited and localized on some roots or invasive to the entire root system, causing wilting of leaves (Figure 1B). Almost all of inoculated plantlets (99.3%) and all genotypes showed symptoms of root rot. The overall mean level of root rot was 4.80 ± 0.12 (Table 2). Figure 1C shows a healthy root and collar root while Figure 1D shows an example of level 5 on the scale for root rot: large roots with few lesions of less than 2.5 cm, as described by Miranda-Fontaíña et al. (2007). Observation in detail (Figure 1G) shows that, at level 5, the lesions on the roots were not continuous. Genotype mean levels ranged from 2.69 (SC51) to 5.67 (SC32) (Table 3). At the time of death, SC51, SC24 and SC57 genotypes displayed confined, small lesions in a few roots, showing resistance to the spread of the pathogen through the roots. These genotypes are significantly different from SC32, SC916, SC919 and SM919 genotypes, which exhibited a high level of root rot, indicating that the pathogen invaded the entire root system.

Most of the inoculated plantlets exhibited root collar rot (84.7%), which an overall average score of 4.70 ± 0.24 on the root collar rot scale (Table 2) and genotype mean levels ranging from 1.93 (SC51) to 6.00 (SC32) (Table 3). Although the genotypes SC32, SC919 and SM919 again showed the highest levels of rot, no significant differences were observed among the genotypes, except for SC55.

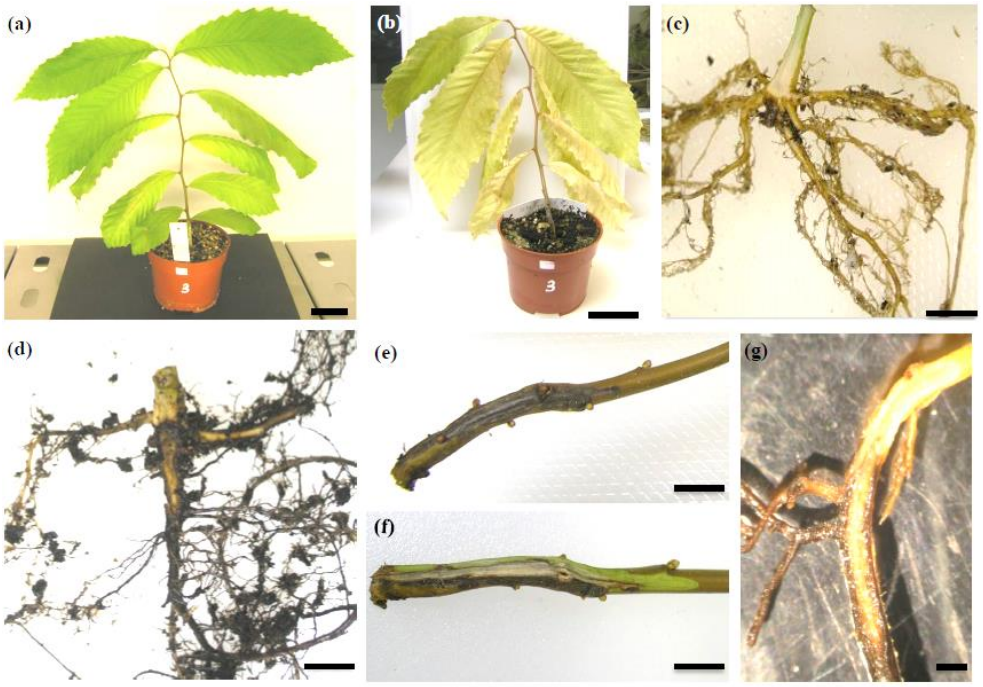

Figure 1. Root inoculated plantlet (SC914, replica 3) at the time of inoculation (a) and after death (b), showing typical symptoms of *P. cinnamomi*. (c) Healthy root and root collar from a control plantlet, without inoculation. (d) Root with high level of root rot (level 5) and low level of collar rot (level 2). (e) External lesion of the longest shoot and (f) internal lesion of the longest shoot. (g) Detail of a fine root with partial root rot. Bars size: a and b= 5 cm; c and d= 2.5 cm; e and f= 1cm and g= 4 mm.

The shoot internal (Figure 1F) and external (Figure 1E) lesions developed in 81.8% and 73.7% of inoculated plantlets, respectively, with mean lesion lengths (as % of shoot length) of 69.45 ± 6.51 and 16.73 ± 2.01, respectively (Table 2). Similar to results obtained for root rot, shoot internal and external lesions were found at their highest levels in genotypes SC32, SC919 and SM919 (Table 3). However, depending on genotype, a large variation was observed in the mean shoot internal lesion, ranging from 14.58% (SC55) to 100% (SC918). The average variation of the percentage of shoot external

lesion was lower than shoot internal lesion, ranging from 4.16% (SM901) to 26.63% (SC918) (Table 3).

The phenotypic and genetic correlation coefficients were estimated for all the variables recorded on inoculated plantlets. Both correlation coefficients showed a great similarity among pairs of variables, in regard to direction and to magnitude (Table 4).

The phenotypic correlations showed that survival had highly significant negative correlations with three of the four ink disease symptoms analysed: level of root collar rot, shoot internal lesion and shoot external lesion. Both phenotypic and genetic correlations showed that shoot internal lesion was the main symptom negatively associated to survival. The level of root rot was the least important symptom associated to survival, with nonsignificant phenotypic correlation (Table 4).

The phenotypic correlations evaluated between survival and the biomass parameters were positive and highly significant (Table 4). Correlations were lower for leaves and shoots than for roots, while the symptoms and biomass parameters were not strongly correlated. The phenotypic correlation coefficients observed between symptoms were positive and highly significant, especially level of root collar rot with shoot external lesion, followed by shoot external lesion with shoot internal lesion. The weakest correlated symptoms were level of root rot with shoot external lesion.

The highest genetic correlations were found among symptoms and among biomass parameters. The genetic correlation coefficients among symptoms ranged from 0.95 to 1.00 (level of root collar rot with shoot external lesion), in agreement with phenotypic correlations.

Table 4. Phenotypic (above the diagonal) and genetic (below the diagonal) correlation coefficients determined between pairs of the variables measured in root inoculation test (N=108 to 137).

	Days of survival	Leaf and shoots dry weight	Root dry weight	Level of root collar rot	Level of root rot	Shoot external lesion	Shoot internal lesion
Age	0.31***	0.38***	0.60***	-0.01	-0.02	-0.05	-0.22*
Days of survival		0.43***	0.50***	-0.42***	-0.16	-0.44***	-0.69***
Leaf and shoots dry weight	0.51		0.75***	0.02	0.18	-0.13	-0.26*
Root dry weight	0.64	0.99		0.02	0.18	-0.10	-0.24*
Level of root collar rot	-0.88	-0.08	-0.23		0.49***	0.76***	0.61***
Level of root rot	-0.86	-0.05	-0.21	1.00		0.40***	0.44***
Shoot external lesion	-0.90	-0.13	-0.29	1.00	1.00		0.66***
Shoot internal lesion	-0.97	-0.36	-0.50	0.96	0.95	0.97	

Phenotypic correlations were analysed with the Spearman's correlation coefficient. Genetic correlations were adjusted for the covariate Age effect (Age=80 days after acclimatization). Significance is indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

The heritability values for chestnut resistance to *P. cinnamomi* varied between 0.34 and 0.90, with low standard errors. Survival showed the highest heritability (0.90 ± 0.04) with low residual variance and thus the highest potential to be inherited. Among symptoms, the highest heritability value was obtained for shoot internal lesion whereas shoot external lesion showed the lowest value, with the lowest variance explained by both components, genetic and residual (Table 5).

Table 5. Narrow-sense heritabilities (h^2) and their standard errors (in parentheses) estimated for the variables (root inoculation test).

Variables	Variance components		Narrow-sense heritabilities (h^2)
	Additive genetic (σ_a^2)	Residual (σ_e^2)	
Days of survival	0.92 (0.34)	0.10 (0.01)	0.90 (0.04)
Level of root rot	0.03 (0.02)	0.04 (0.01)	0.46 (0.16)
Level of root collar rot	0.25 (0.11)	0.22 (0.03)	0.54 (0.11)
Shoot internal lesion	0.45 (0.19)	0.15 (0.02)	0.75 (0.09)
Shoot external lesion	0.01 (0.01)	0.03 (0.00)	0.34 (0.14)

n=108-137.

Excised shoots inoculation

At 5 dai in both seasons, *P. cinnamomi* had induced visible necrotic lesions of varying length, depending on the genotype. At 14 dai a very low percentage (0.36%) of shoots did not show any lesion and 8.80% of shoots showed total necrosis (100% of lesion length).

Analysis of variance revealed that lesion length was significantly affected by Genotype, which had the strongest effect, and also by Season and the interaction of both. In contrast to diameter, the Initial Shoot Length had a significant effect on the lesion length, and therefore was used as a covariate effect in the analysis of SC and SM data.

Further analysis showed that differences observed between genotypes for lesion length were very high for all time points of measurements and in both

seasons, when applicable. Moreover, differences observed between genotypes (SC and SM progenies) for lesion length were maximal at 5 dai (F=19.80, $P < 0.001$) in spring (F=33.11, $P < 0.001$) after bud burst.

Different responses to *P. cinnamomi* were observed in the progenies: a continuous range of resistance-susceptibility levels among genotypes was observed. For the majority of genotypes, the lesion length in the shoots increased over time. In addition, for the most resistant genotypes the lesion length stopped at a given time point, until the end of the experiment. Therefore, the lesion progression rate (cm/day) was calculated for each genotype. Genotype mean values (SC and SM), estimated across the two seasons and adjusted for the covariate Initial Length, are shown in Figure 2. The lesion progression rate varied from 0.15 to 1.13 cm per day across genotypes and seasons; SC57 was the most resistant genotype while SC915 the genotype with the greatest lesion progression rate (i.e. most susceptible). Concerning BC progenies, lesion progression rates obtained in autumn 2015 were also ranged from the most susceptible (BCC01) to the most resistant (BDC40) (Supplementary material 1). This population seems to be more resistant to *P. cinnamomi* than SC and SM, since lesion progression rate varied from 0.11 to 0.89 cm per day.

Correlations between inoculation tests

The estimation of phenotypic and genetic correlations between the root inoculation test and excised shoots inoculation test was possible using the mean response of common genotypes in both tests (n=17). In this way, some differences were obtained in the phenotypic and genetic correlations between survival and symptoms (Table 4 and 6).

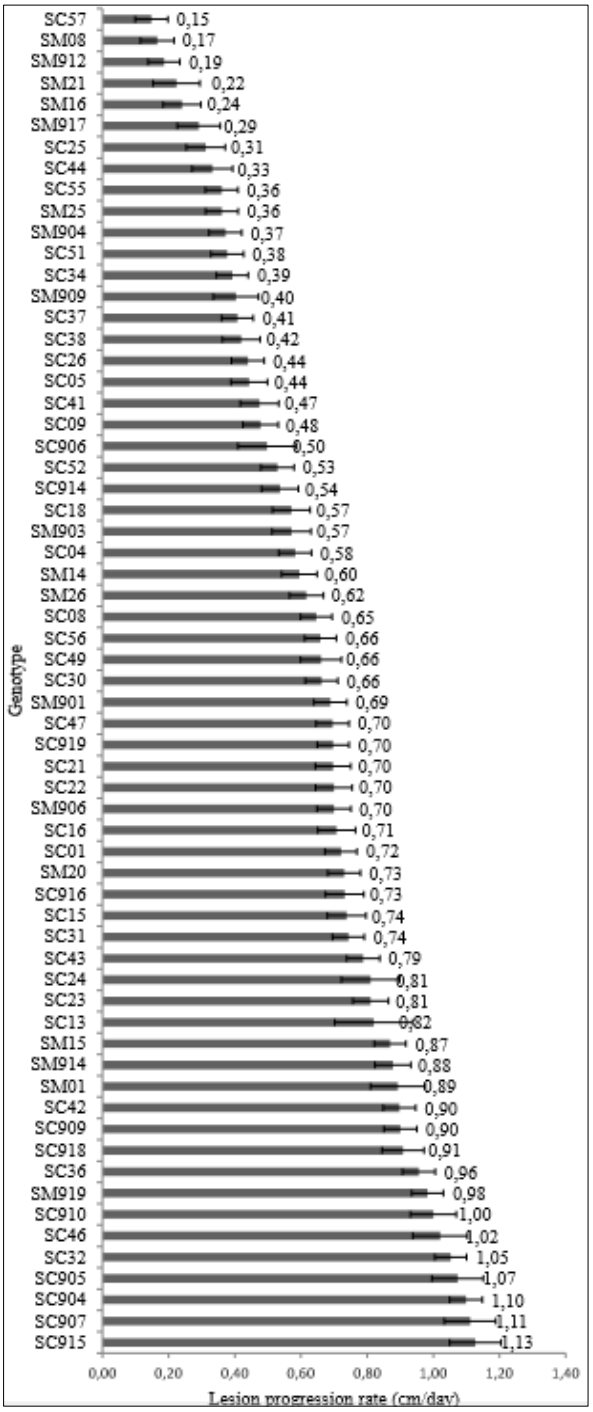

Figure 2. Mean values in lesion progression rate, adjusted for the covariate Initial Length, for 63 genotypes tested with excised shoot inoculation. The genotypes were ranked by lesion progression rate, from the most resistant to the most susceptible. SC915 genotype showed the highest susceptibility to *Phytophthora cinnamomi* whereas SC57 was the most resistant. Bars represent standard errors.

Phenotypic correlations obtained among all variables from both inoculation tests showed that lesion progression rate was the parameter with the strongest correlation with survival (r= -0.85, $P < 0.001$). Shoot internal lesion was correlated moderately with lesion progression rate, as well as level of root collar rot (Table 6). Genetic correlation coefficients were in agreement with phenotypic correlations and the heritability for lesion progression rate was 0.67 ± 0.04.

Table 6. Phenotypic (above the diagonal) and genetic (below the diagonal) correlation coefficients determined between pairs of the variables measured in root inoculation test: Days of survival, Level of root collar rot, Level of root rot, Shoot external lesion and Shoot internal lesion; and between the variables measured in root inoculation test and Lesion progression rate from excised shoot inoculation test (n=17).

	Days of survival	Level of root collar rot	Level of root rot	Shoot external lesion	Shoot internal lesion	Lesion progression rate
Days of survival		-0.63**	-0.36	-0.47	-0.74**	-0.85***
Level of root collar rot	-0.67		0.59*	0.58*	0.56*	0.62*
Level of root rot	-0.56	0.82		0.09	0.36	0.44
Shoot external lesion	-0.45	0.62	-0.05		0.75***	0.45
Shoot internal lesion	-0.87	0.44	0.39	0.66		0.67**
Lesion progression rate	-0.83	0.62	0.73	0.34	0.75	

Significance is indicated by asterisks: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

Discussion

This study addresses two types of inoculation test used to determine the response of individual chestnut plantlets from three mapping populations to inoculation with *P. cinnamomi*. Lesion progression rate was the variable selected to perform DNA marker–variable association, for QTL identification (Chapter V).

Although more than one isolate of *P. cinnamomi* is commonly used in this type of study, only one isolate was used in the present investigation in order to maximize the number of replicates of each chestnut genotype screened, thus making the analysis more robust and accurate. Several previous studies of *P. cinnamomi* on chestnut (Abreu et al. 1999; Dinis et al. 2011) enabled the most virulent isolate to be selected for the present investigation. Frampton et al. (2013) also used a single isolate of *P. cinnamomi* in soil inoculation of *Abies* spp. seedlings. Moreover, Fernández-Lóopez et al. (2001) and Miranda-Fontaiña et al. (2007) observed that there was no significant interaction between isolates and genotypes in the rot symptoms evaluated, indicating no specificity of those isolates in chestnut.

In previous studies, the origin and physiological conditions of plant material, replicate number, time point of lesion measurements and test conditions varied for both inoculation tests (Vettraino et al. 2001a,b; Robin et al. 2006; Miranda-Fontaiña et al. 2007; Cuenca et al. 2009). Therefore, in the present study, the experiments were designed in order to obtain the most reliable results possible: a high number of clonal plantlets per genotype and the use of the same controlled environmental conditions for both inoculation tests. Clonal testing of progeny from mapping populations is the most efficient way to minimize the effect of environmental variation and obtain better estimates of the phenotypic value (Bradshaw & Foster 1992). Minimizing environmental variation and therefore increasing heritability, increases the robustness and the ability for QTL detection.

In the root inoculation test, control plantlets grew more than inoculated plantlets, as expected. The lesions in roots and shoots caused by *P. cinnamomi* may hinder water and nutrient absorption, and as a consequence, cause a reduction in the photosynthesis rate and growth. Robin et al. (2006) and Miranda-Fontaiña et al. (2007) also reported a reduction in growth of chestnut plants inoculated with *P. cinnamomi*. Cahill et al. (1989) observed

that *P. cinnamomi* inoculation stopped root growth in a group of plant species within 24–48 h.

Previous studies have indicated that days of survival should be the main discriminator of *Phytophthora* spp. resistance in chestnut (Vettraino et al. 2001a), in *Abies* spp. (Frampton et al. 2013) and in *Eucalyptus* spp. (Stukely & Crane, 1994). However, other authors have considered the level of root or collar rot as the main indicator of resistance to *Phytophthora* spp. in chestnut (Robin et al. 2006; Miranda-Fontaiña et al. 2007; Cuenca et al. 2009). The present study showed that variable 'Days of survival' was the most important indicator of resistance to *P. cinnamomi* because differences in response between genotypes were maximized; the presence of high levels of root and collar rot in almost all inoculated plantlets indicated that these symptoms were not good discriminators for resistance. Miranda-Fontaiña et al. (2007) also reported high levels of root and collar rot in a high percentage of chestnut plants. Likewise, Cuenca et al. (2009) observed root rot in 60% of the resistant *C. crenata* plants. Survival has not been considered as the main descriptor of resistance to *P. cinnamomi* in chestnut, mainly because of the high mortality of control chestnut plants before and during the experiment, due to biotic or abiotic factors or cross contamination (Miranda-Fontaiña et al. 2007; Cuenca et al. 2009). In this study, all control plantlets survived until the end of the experiment, indicating that *P. cinnamomi* cross contamination and other biotic and abiotic stresses, such as flooding, did not occur. In future, similar studies should include preventive measures, such as the use of sterile substrates and avoiding excess flooding time during the experiments. Twenty to thirty minutes flooding is sufficient for *P. cinnamomi* release zoospores and cysts to germinate (Hardham, 2005). In summary, long survival was considered evidence of high resistance and thus seven genotypes (35%) were selected as the most resistant.

In this study, shoot internal lesion was evaluated for the first time as a parameter to assess chestnut resistance to *P. cinnamomi*. It was chosen because it indicates the spread of the pathogen from the roots and collar to the aerial vascular system. This is important for determining the degree of plant resistance, as the rapid invasion of the pathogen into the phloem and xylem may affect water and nutrient movement through the shoots, causing death.

With regard to biomass parameters, the phenotypic correlation was stronger between survival and root dry weight than with leaf and shoot dry weight. Cuenca et al. (2009) also observed a good correlation between fresh root weight and survival. A healthy and developed root system is an important factor for resistance to *P. cinnamomi*.

The lesion caused by the inoculation of excised shoots is considered to be an indirect measure of *Phytophthora* spp. resistance. The length of the lesion is negatively proportional to resistance to the pathogen (Fernández-López et al. 2001). The results showed that, similar to root-inoculated plants, the resistance to *P. cinnamomi* in the shoots is related to the confinement of the lesion to point of inoculation. For the most resistant genotypes, the surrounding tissues dried, limiting the progression of the lesion.

In the present study, differences observed between genotypes for lesion length were very high for all time points of measurement and in both seasons. Nevertheless, the results revealed that spring was the better season to perform excised shoot inoculation tests. After budburst (in spring), plants have good physiological conditions that may allow a better resistance response. It was found that the best time to take measurements in future investigations would be 5 dai, when differences in lesion lengths between genotypes were maximized.

The present study has shown that the strongest and most significant phenotypic and genetic correlations were obtained for lesion progression

rate and survival; therefore, these would be the best variables to measure in future investigations.

The estimation of heritabilities and genetic correlations (genetic parameters) is an important strategy for plant breeding. Phenotypic variables with higher heritabilities (in this study: survival, shoot internal lesion and lesion progression rate) have the potential to be inherited to varying degrees in populations exposed to differential natural selection pressures in distinct environments (White et al. 2007).

Resistance to *P. cinnamomi* is a polygenic and quantitative trait (Irwin et al. 1995) that was here evaluated by measuring several variables. The strong genetic correlations observed between survival and symptoms suggest common genetic determinants. Similarly, survival had a strong genetic correlation with lesion progression rate in the excised shoot inoculation test. The assessment of survival by root inoculation testing is expensive and laborious and cannot always be determined in a population. The strong favourable genetic correlation observed between the two variables suggests that indirect selection could be made by lesion progression rate, which is easily measured. Therefore, the *C. sativa* x *C. crenata* population obtained in 2015 (BC) was phenotyped using the excised shoot inoculation test in autumn. Preliminary results indicate a higher level of resistance when compared with SC and SM populations. However, excised shoot inoculation test shall be repeated in spring to obtain lesion progession rates across both seasons. Then, mother plants will be root-inoculated with *P. cinnamomi* to identify the most resistant genotypes (survivors to the inoculation). Finally, those genotypes will be established for *in vitro* culture for further phenotype validation by root inoculation test.

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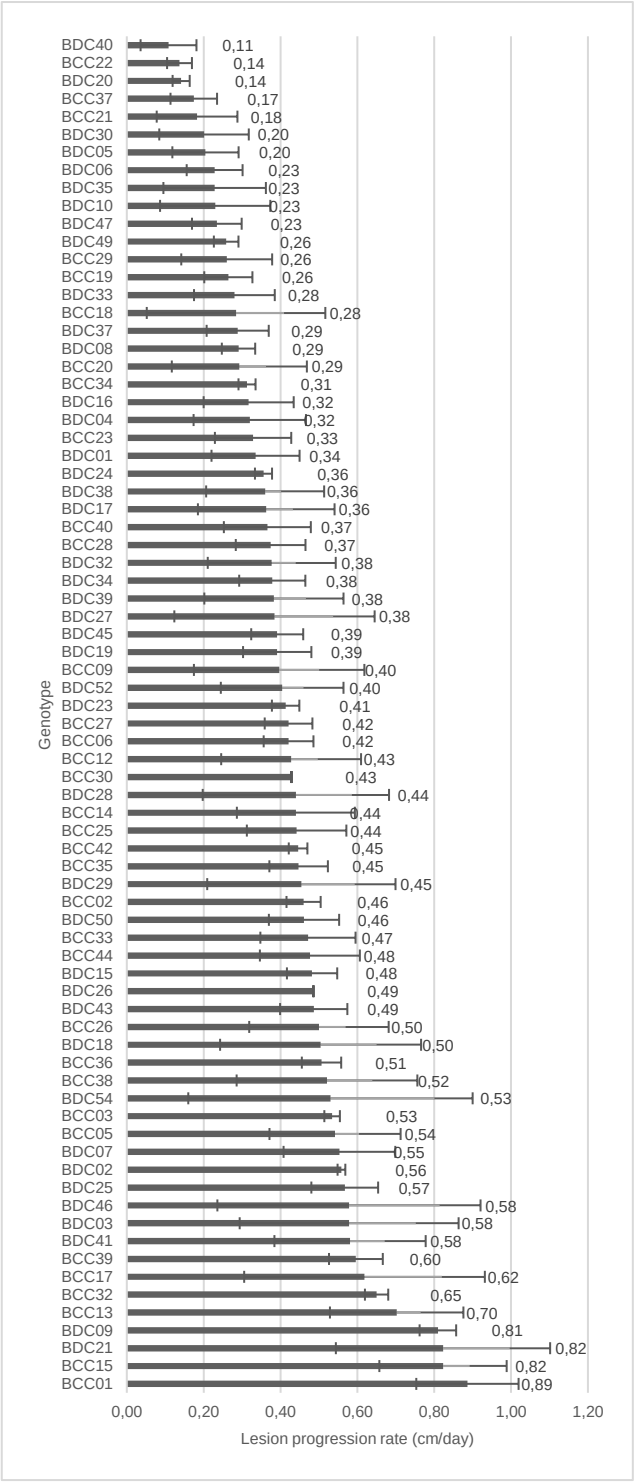
Supplementary material



Supplementary material 1. Mean values in lesion progression rate, for 76 (population BC) genotypes tested with excised shoot inoculation. The genotypes were ranked by lesion progression rate, from the most resistant to the most susceptible. BCC01 genotype showed the highest susceptibility to *Phytophthora cinnamomi* whereas BDC40 was the most resistant. Bars represent standard errors.

Chapter III

Castanea root transcriptome in response to *Phytophthora cinnamomi* infection

The work presented in this chapter was published in the following research publication:

Serrazina S., Santos C., Machado H., Pesquita C., Vicentini R., Pais M.S., Sebastiana M. and Costa R. (2015) *Castanea* root transcriptome in response to *Phytophthora cinnamomi* challenge. *Tree Genet. Genomes* 11, 6. doi: 10.1007/s11295-014-0829-7

In this research paper Carmen Santos participated in the experimental design, pathogen inoculations, RNA isolation and paper writing.

Abstract

The European chestnut, an important forest species for the economy of Southern Europe, covers an area of 2.53 million hectares, including almost 110 000 hectares devoted to fruit production. *Castanea sativa* is declining due to ink disease caused by *Phytophthora cinnamomi*. To elucidate chestnut defense mechanisms to ink disease we compared the root transcriptome of the susceptible species *C. sativa* and the resistant species *C. crenata* after *P. cinnamomi* inoculation. Four cDNA libraries were constructed, two of them included root samples from *C. sativa*, inoculated and non-inoculated and the other two libraries comprised samples from *C. crenata* at identical conditions.

Pyrosequencing produced 771 030 reads and assembly set up 15 683 contigs for *C. sativa* and 16 828 for *C. crenata*. GO annotation revealed terms related to stress as 'response to stimulus', 'transcription factor activity' or 'signaling' for both transcriptomes. Differential gene expression analysis revealed that *C. crenata* involved more genes related with biotic stress upon pathogen inoculation than *C. sativa*. Those genes for both species are involved in regulation of plant immune response and stress adaptation and recovery. Furthermore, it is suggested that both species recognize the pathogen attack; however, the resistant species may involve more genes in the defense response than the susceptible species. RNA-seq enabled the selection of candidate genes for ink disease resistance in *Castanea*. The present data is a valuable contribution to the available *Castanea* genomic resources and constitutes the basis for further studies.

Keywords: *Castanea sativa*; *Castanea crenata*; *Phytophthora cinnamomi*; RNA-seq; differentially expressed genes; biotic stress.

Introduction

European chestnut (sweet chestnut, *Castanea sativa* Miller) has great economic value due to fruit production, and ecological value including forest diversity and soil stability. In the last 100 years, ink disease caused by the soil oomycete *Phytophthora cinnamomi* Rands has contributed to a drastic reduction of *C. sativa* distribution area in Europe. Oomycetes (eukaryotic heterokonts) show strategies of plant infection that are similar to many fungal pathogens (Latijnhouwers et al. 2003). Sweet chestnut groves have also been affected since the 1930's by the chestnut blight fungus [*Cryphonectria parasitica* (Murril) Barr], causal agent of the American chestnut [*Castanea dentata* (Marshall) Borkh.] decimation.

Ink disease was introduced to Europe from the USA through the Azores islands (Fernandes 1955; Anagnostakis 2001). The first records on its appearance in northern Portugal date from 1838. It has since been reported in many European countries, including Spain, Italy, France and the United Kingdom. The progression of the disease in grove areas with high humidity has limited the establishment of new groves and impeded the conservation of old ones (Vannini and Vettraino 2001). Presently, the greatest impact of ink disease is limited to the warm southwestern and southern regions of central Europe [reviewed by (Brasier and Jung 2006)]. In the 19th century, ink disease was partially responsible for a decline of *C. dentata* in the Southeastern USA (Anagnostakis 2001), prior to its broad decimation by chestnut blight. Ink disease is currently re-emerging in the USA and constitutes a serious threat to the American chestnut reintroduction (Jacobs et al. 2013).

Common woody hosts of *P. cinnamomi* include *Eucalyptus*, *Quercus*, *Juglans*, *Betula* and *Castanea*, and the mycelia also persist saprophytically in soil. In the presence of water, oospores and chlamydospores differentiate sporangia that form and release zoospores. Zoospores are motile and are able to penetrate non-lignified root tissue and the base of stems or trunks:

both scenarios result in local tissue rot. Growth, reproduction and dissemination of the pathogen are favored under compacted and water saturated soils with poor aeration. Symptoms on the adult trees include leaf chlorosis, thinning of the crown and the persistence of immature fruits on the trees after leaf-fall. Larger roots are mainly affected, producing a black exudate which increases during spring and fall. Infected seedlings undergo a rapid or gradual leaf wilting, depending on the severity of the infection. The root system suffers extensive necrosis of the tap root that extends to the lateral roots and up the lower stem (Vannini and Vettraino 2001). Oßwald et al (Oßwald et al. 2014) explain the primary physiological, biochemical and molecular reactions described on infected roots of susceptible *Phytophthora*-host interaction, summarized as follows: 1) The pathogen releases elicitins into the rhizosphere, facilitating root penetration; 2) Down-regulation of defense genes in the host, facilitating pathogen growth; 3) Destruction of roots and impairment of water and nutrient uptake; 4) Increase of the abscisic acid phytohormone in roots; 5) Decrease in leaf water potential; 6) Stomata closure and decrease in photosynthesis; 7) Probable release of toxins and effectors into the host tissue during biotrophic growth of the pathogen and transport into the canopy via xylem sap flow; 8) Up-regulation of genes of the ethylene pathway and release of the phytohormone by leaves; 9) Decrease in cytokinin content in roots during the necrotrophic growth of the pathogen; 10) Chlorosis and wilting of leaves resulting from the changed water and hormonal status of the host caused by root infection.

Progression of ink disease depends on environmental conditions, pathogen virulence and plant susceptibility. One strategy to control the disease is through breeding with resistant species. Soon after the introduction of Asian chestnuts to Europe it was verified that *C. crenata* (*Castanea crenata* Siebold & Zucc., the Japanese chestnut) has a high level of resistance to *Phytophthora* (Vannini and Vettraino 2001). Since the 1950's, breeding programs with the European and Japanese chestnut were established in

Portugal, France and Spain to obtain hybrids tolerant to ink disease, while maintaining fruit production and quality traits to satisfy commercial demands (Vannini and Vettraino 2001; Martins et al. 2009). However, fruit quality produced by these hybrids is below current market standards, so there is demand from both researchers and producers, for developing genomic tools to understand resistance mechanisms against *P. cinnamomi*. Barakat et al (Barakat et al. 2009; Barakat et al. 2012) described the generation of more than 1,5 million cDNA sequences for the American and Chinese chestnuts that have been used to analyse chestnut resistance to *C. parasitica*. The data are available through the Fagaceae Genomics Web (http://www.fagaceae.org/) and represent the first public resource on chestnut transcriptomes. The data we present here contribute to this resource by identifying Japanese and European chestnut genes involved in the reaction to ink disease, another critical threat to *Castanea*.

To compare the response of the resistant Japanese chestnut with the response of the susceptible European chestnut to *P. cinnamomi* infection, four cDNA libraries of *C. sativa* (Cs) and *C. crenata* (Cc) root tissues, inoculated (i) and non-inoculated (n) with the pathogen were prepared for 454 pyrosequencing. Contig annotation and analysis of transcript abundance supported the quantification of transcript expression on inoculated and non-inoculated roots in each species, as well as, identifying differentially expressed genes upon pathogen inoculation. This allowed a comparison of each species' response to the pathogen and the selection of candidate genes for resistance to ink disease.

Materials and Methods

Plant material and pathogen inoculation

The TRAGSA nursery (Grupo TRAGSA-SEPI, Maceda, Spain) provided 36 micropropagated plants at five years of age, 18 of *C. sativa* (Cs, susceptible) and 18 of *C. crenata* (Cc, resistant). Four treatments were set, corresponding

to *C. sativa* and *C. crenata* inoculated and non-inoculated with *P. cinnamomi* (Supplementary material 1). Plants were distributed in 15 L pots with peat.
A hypervirulent isolate of *P. cinnamomi* (IMI 340340) provided by Trás-os-Montes and Alto Douro University was grown at 22°C on Potato Dextrose Agar. For soil infestation *P. cinnamomi* inoculum was prepared by growing mycelia on sterilized millet seeds (*Ponicium mileaceum*), which were thoroughly moistened with vegetable juice (V8®) broth [20% (v/v) with 3 g/L of $CaCO_3$]. The mixture was incubated for three weeks in darkness at 24°C. At the time of inoculation (0 h), *P. cinnamomi* was carefully added to each container substrate at a concentration of 5% (v/v), in order to minimize root disturbance and wounding. No pathogen was added to non-inoculated plants. After inoculation all containers were flooded for 3h to stimulate zoospore release and to promote disease development. At 2, 4 and 7 days after inoculation, 6 plants per treatment (3 of *C. sativa* and 3 of *C. crenata*, Supplementary material 1) were removed from containers and root samples were collected. After rinsing, roots were frozen in liquid nitrogen and stored at -80°C.

RNA isolation

Total RNA from root tissue was isolated based on Le Provost et al (Le Provost et al. 2007). RNA integrity and purity was determined with a 2100 Bioanalyser with the RNA 6000 Pico kit (Agilent Technologies, Palo Alto, CA, USA). In order to compare gene expression between the two chestnut species after pathogen inoculation, four RNA pools were prepared, based on the experimental design described by Barakat et al (Barakat et al. 2009; Barakat et al. 2012): Cci, Ccn, Csi and Csn (i: inoculated; n: non-inoculated). Each pool included the RNA from nine plants, 3 biological replicates collected at 3 time points after inoculation (2, 4 and 7 days, Supplementary material 1).

Poly(A) RNA enrichment, cDNA library construction and pyrosequencing

The procedures described in this section were provided by the Next Gen Sequencing Unit at Biocant (Cantanhede, Portugal).

The integrity of all RNA pools was verified on a 2100 Bioanalyser as above and the quantity assessed by fluorometry with the Quant-iTRiboGreen RNA kit (Invitrogen, CA, USA). Poly(A)RNA was enriched from total RNA using two rounds of the MicroPoly(A) Purist Kit (Applied Biosystems, Ambion, CA, USA), according to the manufacturer's instructions. The RNA quality was again assessed on a 2100 Bioanalyser and the quantity determined by fluorometry as described above.

A fraction of 200 ng of Poly(A)+ RNA of each isolate was used as starting material for cDNA library construction using Multiplex Identifiers (MIDs) according to the cDNA Rapid Library Preparation Method Manual*,* 'GS FLX Titanium Series, October 2009' (Roche-454 Life Sciences, Brandford, CT, USA). The four dscDNA libraries were quantified by fluorescence, pooled in equimolar amounts and pyrosequenced in a single plate with GS FLX Titanium chemistry (Roche-454 Life Sciences, Brandford, CT, USA), according the standard manufacturers' procedures.

Transcript assembly and functional annotation

After 454 sequencing, the raw reads were processed to remove sequences with less than 100 nucleotides and low quality regions. Ribosomal, mitochondrial and chloroplast reads were identified through BLASTx against the non-redundant NBCI database and any hits with an *E* value of 0.0 were removed from the data set. All remaining reads were then assembled into contigs using 454 Newbler 2.6 (Roche, Branford, CT, USA) with the default parameters (40 bp overlap and 90% identity). A three step analysis was carried out to identify genes. First, the translation frame of each contig was assessed through BLASTx searches against Swissprot (*E* value<1E-6) and the corresponding amino acid sequence was translated using an in-house

script. Then any contigs without translation were submitted to FrameDP (Gouzy et al. 2009) software with default parameters. Finally, all remaining contigs were analysed with ESTScan (Lottaz et al. 2003) with default parameters. Transcripts identified by FrameDP or ESTScan were searched using BLASTp against the non-redundant NBCI database (*E* value<1E-2) to translate putative proteins. The functional annotation of all translated amino acid sequences was predicted through assignment into protein families and identification of protein domains using InterProScan version 4.6 (Hunter et al. 2009). Gene Ontology (GO) terms identified by InterProScan results for each translated amino acid sequence were additionally retrieved and added to classify each transcript product. The procedures above described were provided by the Next Gen Sequencing Unit at Biocant.

All contigs were taxonomy annotated in order to separate the sequences belonging to the *Streptophyta* phylum for further analysis. To obtain the taxonomical assignments we uploaded the contigs to MG-RAST (Meyer et al. 2008) (with default parameters), an automated analysis platform for metagenomes based on sequence similarity to both protein and nucleotide databases.

Identification of differentially expressed genes related to *P. cinnamomi* resistance

Differentially expressed genes were identified as genes showing significant higher/lower expression levels in inoculated root tissue versus non-inoculated root tissue. The number of reads mapping to each transcript (contig) in the two treatments (inoculated and non-inoculated) was counted and used as an approximate estimation of gene expression level in the corresponding tissues. First, the contigs from the different samples were clustered at 90% similarity and 95% identity by CD-Hit 454 (Niu et al. 2010) to eliminate redundant sequences and generate reference contigs. The reads from each sample were then mapped to those references with Newbler

mapping 2.6 (Roche, Branford, CT, USA) using the default parameters, and the number of reads contributed by each sample counted. Reads with multiple hits were discarded. The number of reads per reference contig per sample was used to build a contingency table, which was analysed with the Myrna statistical analysis package (Langmead et al. 2010), with the normalization factor set to 95th percentile. Statistical significance of the differential expression was evaluated using a linear regression model based on a Gaussian distribution, and using only contigs with a minimum of eight mapped reads. All results were compiled into a SQL database developed as an information management system. The procedures described above were provided by the Next Gen Sequencing Unit at Biocant.

For the selection of Differentially Expressed Genes (DEGs), contigs with a *P* value <1E-03 were considered. *P* value describes the probability that differences in counts between the two sets in comparison are due to chance (Langmead et al. 2010). Fold expression changes were calculated for the inoculated vs. non-inoculated comparisons, Csi-Csn and Cci-Ccn. Contigs with a fold change greater than 1 were classified as up-regulated genes and contigs with a fold change less than to 1 were classified as down-regulated genes. For further analysis of DEGs (*P. cinnamomi* resistance-related genes), the $\log_2$ of fold change>|1| criteria was applied.

DEGs were also analysed for the two inoculated species in the comparison Csi-Cci (*P* value<1E-03) to reveal the genes that were significantly induced in both species after pathogen challenge. On the other hand, the comparison of the two non-inoculated species Csn-Ccn (*P* value<1E-03) revealed the constitutive genes in both species whithout inoculation.

The application Blast2GO (Conesa and Götz 2008), namely the Enrichment Analysis, was used to statistically analyse GO annotation in the comparisons Csi-Csn, Cci-Ccn, Csi-Cci and Csn-Ccn for DEGs. It employs a Fisher's exact test with multiple testing correction of FDR (Benjamini and Hochberg). Upon selection of a single test and *P* value <5E-3, all GO terms were tested

if they are enriched in the DEGs group when compared to a reference group (all contigs in the comparison).

454 sequencing validation by real-time PCR

The relative expression of a subset of genes was achieved by quantitative real-time PCR (qRT-PCR) to validate RNA-sequencing (RNA-seq). The four RNA pools used for sequencing (Cci, Ccn, Csi and Csn) were prepared for qRT-PCR as follows: RNA was treated with DNase (Turbo DNase-free kit Ambion, Inc., USA), according to manufacturer's instructions. cDNA was synthesized using RevertAid H Minus Reverse Transcriptase (Thermo Fisher Scientific, Waltham, USA) according to manufacturer's instructions. Gene specific primers were designed for six target genes (Supplementary material 2) using Primer Express (version 1.0, Applied Biosystems, Sourceforge, USA). *Actin-7* was selected as a reference gene after verifying a similar number of reads for all cDNA libraries and used for normalization of expression. A final concentration of 0,2 µM of each primer was used in 25 µL reactions, together with cDNA as template and Maxima SYBR Green/ROX qPCR Master Mix (Fermentas, Ontario, Canada), on a StepOne™ Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Thermal cycling for all genes started with a denaturation step at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing temperatures for 30 s. Three technical replicates were used per reaction set, including template and no template controls. Non-specific PCR products were analysed by dissociation curves. The relative expression value and mean absolute deviation values were calculated for the pool comparisons Cci-Ccn and Csi-Csn according to the ΔΔCT method (Livak and Schmittgen 2001).

Results

454 sequencing and assembly summary

Two Japanese chestnut cDNA libraries were constructed, one from a RNA pool of inoculated root tissue (Cci) and the other from a RNA pool of non-inoculated root tissue (Ccn). A half plate of sequencing was used, resulting in 220 412 reads for Cci and 182 314 reads for Ccn, with an average read length of 350 nt (Table 1). Approximately 77 and 64 megabases of cDNA were generated for Cci and Ccn respectively. After assembly, 8 528 contigs were generated for Cci and 8 300 contigs were generated for Ccn, with an average length of 885 nt. 2 712 Cci contigs and 2 214 Ccn contigs had more than 1000 nt, corresponding to 32% and 27% of all respective contigs.

Table 1. Summary of 454 sequencing for *Castanea crenata* and *Castanea sativa* root transcriptomes.

cDNA library	**Cci**	**Ccn**	**Csi**	**Csn**
Roots sampled	*C. crenata* inoculated	*C. crenata* non-inoculated	*C. sativa* inoculated	*C. sativa* non-inoculated
No. of plates	¼	¼	¼	¼
No. of reads	220 412	182 314	181 384	186 920
Average read length (nt)	350	350	357	367
No. of bp	77 175 000	63 823 300	64 884 000	68 672 896
No. of contigs	8 528	8 300	7 208	8 475
Average contig length (nt)	915	854	856	823
No. of large contigs[a]	2 712	2 214	1 943	2 065
No. of putative proteins	8 149	7 969	6 852	8 073
AA sequences assigned to InterPro terms	6 373	6 279	5 350	6 213
AA sequences assigned to GO terms	4 885	4 790	4 090	4 691

[a] Greater than 1000 nt

Likewise, two cDNA libraries were also constructed for European chestnut, Csi (inoculated roots) and Csn (non-inoculated roots). A half sequencing plate resulted in 181 384 reads for Csi and 186 920 reads for Csn, with an average read length of 362 nt (Table 1). Csi and Csn libraries contained approximately 65 and 69 megabases, respectively. Assembly resulted in 7208 contigs for Csi and 8 475 contigs for Csn, with an average length of 840 nt. 1 943 Csi contigs and 2 065 Csn contigs had more than 1000 nt, corresponding to 27% and 24% of total respective contigs.

Functional annotation

BLASTx against Swissprot, FrameDP and ESTScan tagged 8,149 Cci contigs and 7 969 Ccn contigs as putative proteins. Likewise, 6 852 contigs were tagged for Csi and 8 073 contigs were tagged for Csn. This indicates that 95% of the contigs could be tagged as putative proteins, comprising highly informative transcripts on a plant genome that has not yet been sequenced.

The *Castanea-P. cinnamomi* system in the present report was analysed on plants in non-sterile soil containers and comprises several other organisms, mostly soil-borne. To analyse *C. sativa* and *C. crenata* metagenomes concerning taxonomic hit distribution, all contigs from the four libraries were uploaded to MG-RAST server. Results were similar for all four root transcriptomes (Cci, Ccn, Csi and Csn) and are combined in Figure 1. Sequence alignments to the M5 non-redundant protein database (M5NR) revealed that *C. crenata* and *C. sativa* contigs have the highest similarities with plant proteomes: *Arabidopsis thaliana* (24%), *Vitis vinifera* (19%), *Ricinus communis* and *Populus trichocarpa* (both 15%). Presently the sequenced genome of *Arabidopsis thaliana* is the one with the highest level of information among the dicotyledonous. *Vitis vinifera*, *Ricinus communis* and *Populus trichocarpa* are woody/semi-woody plants, all with sequenced genomes with similar coverage and taxonomically close to *Castanea*.

Barakat et al (2012) obtained comparable similarities with their proteomes for Chinese and American chestnut transcripts. MG-RAST attributed 13% of the annotated contigs to 'unknown proteins' that may contain chestnut specific proteins. Additionally, 1% of the contigs were classified as 'unknown sequences' that had no homology to predicted proteins or rRNA and may correspond to 5' or 3' untranslated regions and small RNAs. The very low percentage of 'non *Streptophyta*' sequences (4%) may be correlated to the presence of other organisms in the soil as bacteria or fungus (*Ascomycota* and *Basidiomycota*).

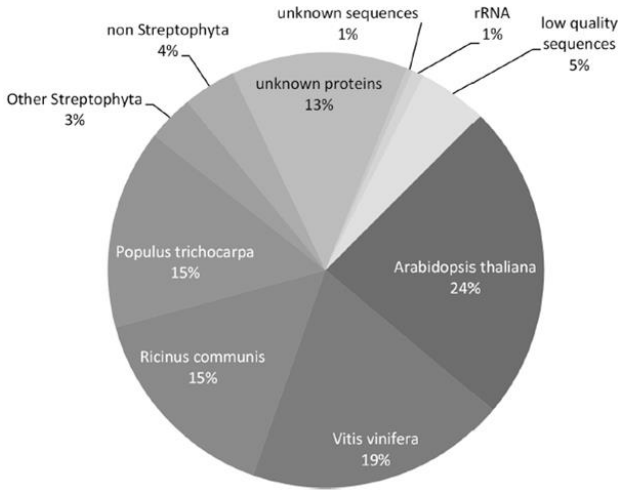


Figure 1. Taxonomic hits of *Castanea crenata* and *Castanea sativa* contigs at the proteome level. All contigs were uploaded to MG-RAST and annotated.

The contigs of all four libraries were uploaded to InterProScan, which provided Gene Ontology (GO) terms within the three GO categories (Molecular Function, Biological Process and Cellular Component) for functional annotation. As exposed in Table 1, GO terms were attributed to 57% of the contigs: 4 885 for Cci, 4 790 for Ccn, 4 090 for Csi and 4 691 for Csn. When the annotation sets of *C. crenata* (Cci, Ccn) and *C. sativa* (Csi, Csn) were combined on Blast2GO, the distribution of GO terms was similar for the two species' root transcriptomes (Figure 2).

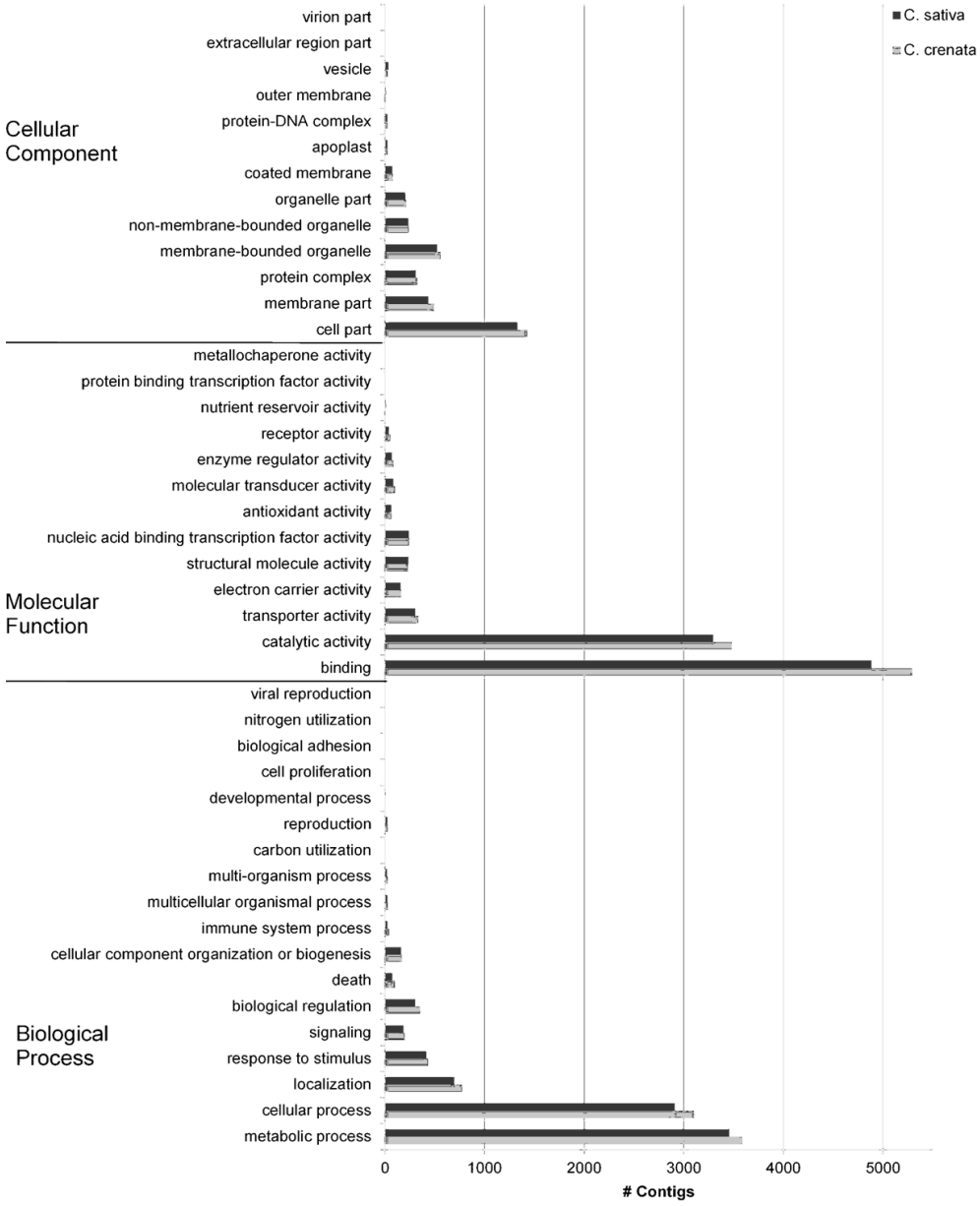


Figure 2. Distribution of *Castanea crenata* and *Castanea sativa* contigs into functional sub-categories of Gene Ontology. Only contigs corresponding to putative proteins were considered in this analysis. GO level: 2 for Biological Process and Molecular Function and 3 for Cellular Component.

'Membrane part' and 'membrane-bounded organelle' are well represented on Cellular Component (CC) category and include genes related to biotic stress regulation and recovery. The Molecular Function (MF) terms 'binding' and 'catalytic activity' include 'hydrolase activity' (with 949 and 200 contigs respectively) and genes involved in the synthesis of enzymes that degrade pathogenic fungal cell walls. Moreover, 'transporter activity' and 'nucleic acid binding transcription factor activity' enclose genes involved in biotic stress signaling and regulation. The Biological Process (BP) 'metabolic process' includes 'oxidation-reduction process' (587 contigs) and 'cellular process' includes 'cell communication' (229 contigs), pointing to genes related to anti-fungal metabolite synthesis and stress signaling, respectively. We also highlight the BP terms 'biological regulation' and 'response to stimulus', which contains 'response to stress' (230 contigs). This GO terms distribution indicates that *C. crenata* and *C. sativa* transcriptomes are suitable for stress physiology studies and for the selection of candidate genes to ink disease resistance.

Differentially expressed genes after pathogen inoculation

RNA-seq read count is directly related to gene expression levels between the two experimental conditions (Langmead et al. 2010). 454 Newbler Mapping 2.6 quantified the reads in each *Castanea* library for a specified contig. Then Myrna attributed statistical significance to the difference between the reads or a *P* value, which was used to select Differentially Expressed Genes after pathogen inoculation (DEGs). *C. crenata* DEGs derive from a comparison of the reads in the pathogen inoculated library to the reads in non-inoculated library (Cci-Ccn). Likewise, *C. sativa* DEGs derive from the comparison Csi-Csn. With a *P* value<1E-3, 283 DEGs could be identified in the Cci-Ccn comparison and 305 DEGs in Csi-Csn comparison (Supplementary material 3 and Supplementary material 4). In inoculated *C. crenata* 229 and 54 contigs were respectively up and down-

regulated relative to non-inoculated roots (Figure 3). After analysis of BLAST best hits, we identified 34% of up-regulated contigs and 6% of down-regulated contigs related with a response to pathogens. In inoculated *C. sativa* 117 contigs were up-regulated and 188 genes were down-regulated relative to non-inoculated root tissue. Eighteen percent of the up-regulated and 10% of the down-regulated contigs were related with biotic stress response.

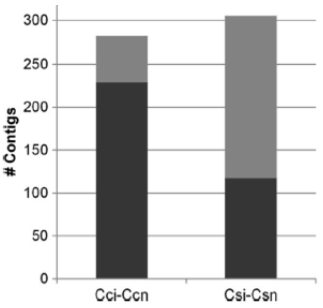


Figure 3. Differentially expressed genes in the root transcriptomes of *Castanea crenata* (Cc, resistant) and *Castanea sativa* (Cs, susceptible), *P* value<1E-3. A contig is up-regulated when the ratio i/n (number of reads in inoculated library/number of reads reads in non-inoculated library) is >1 and down-regulated when the same ratio is <1. Grey: down-regulated contigs; Black: up-reguated contigs.

We analysed GO annotation of DEGs in the comparison sets Cci-Ccn and Csi-Csn and the enrichment analysis revealed divergences for the two chestnut species (Figure 4). Upon inoculation, genes up-regulated in *C. crenata* but not in *C. sativa* included transcription factors (TF) assigned to the GO terms ‘DNA binding’ (MF) and ‘Sequence-specific DNA binding transcription factor activity’ (MF), related to the regulation of the host response to the pathogen (e.g. *WRKY transcription factor 31*) (Zhang et al. 2008b). The term ‘Cell wall’ (CC) also was only revealed for *C. crenata*, with transcripts involved in cellulose synthesis (e.g. *Xyloglucan endotransglucosylase/hydrolase protein 23*) (Yokoyama and Nishitani 2001). The term ‘Carbon-carbon lyase activity’ (MF) was common to up-regulated DEGs in both species, with genes involved in glycolysis (e.g. *Fructose-bisphosphate aldolase, cytoplasmic isozyme 2*). The enrichment analysis of GO annotation for down-regulated DEGs upon inoculation (Figure 4) revealed a common BP term for the two species, ‘Cellular polysaccharide

metabolic process', which includes genes related with starch and cellulose synthesis (e.g. *Granule-bound starch synthase 1, chloroplastic/amyloplastic*). *C. sativa* had a preponderance of down-regulated genes included in the term 'Catalytic activity' (MF), including genes related with the response to oxidative stress (e.g. *Glutamate decarboxylase 1*) (Bouché and Fromm 2004), secondary metabolite synthesis (e.g. *Chalcone--flavonone isomerase 1*) (Hartmann et al. 2005), general defense proteins (e.g. *Pleiotropic drug resistance protein 2*) (Yazaki 2006), receptor-like protein kinases responsive to pathogen infection (e.g. *Cysteine-rich receptor-like protein kinase 10*) (Chen et al. 2004) and jasmonic acid (JA) synthesis (e.g. *Allene oxide synthase*) (Sivasankar 2000). The GO term 'Oxidation-reduction process' (BP) was the most significant for *C. crenata* down-regulated DEGs, comprising genes related to secondary metabolite synthesis (e.g. mevanolate biosynthesis, *3-hydroxy-3-methylglutaryl-coenzyme A reductase*) (Leivar et al. 2011).

The comparison of *C. crenata* and *C. sativa* DEGs (Supplementary materials 3, 4, 5 and 6) revealed common up-regulated genes after inoculation that may be involved in *P. cinnamomi* response (Supplementary material 7). We associated those genes into functional categories and highlight a) Regulation of plant immune response, with four genes including *Lipoxygenase A*, related to signaling during pathogen attack and induction of cell death (Porta and Rocha-Sosa 2002), and b) four genes related to drought stress, e.g. *Dehydrin DHN2 (Yang et al. 2012)*. In the comparison of Cci-Ccn with Csi-Csn we also identified one common down-regulated DEGs, *Zinc finger protein1 WZF 1*, a probable transcriptional repressor (Ohta et al. 2001).

Further analysis of the data found in Supplementary materials 3, 4, 5 and 6 revealed *C. sativa* down-regulated genes that correspond to up-regulated genes in *C. crenata*. These transcripts were associated in the functional categories Regulation, Lipid signaling, Anti-fungal metabolite synthesis, Cell wall synthesis and Stress recovery.

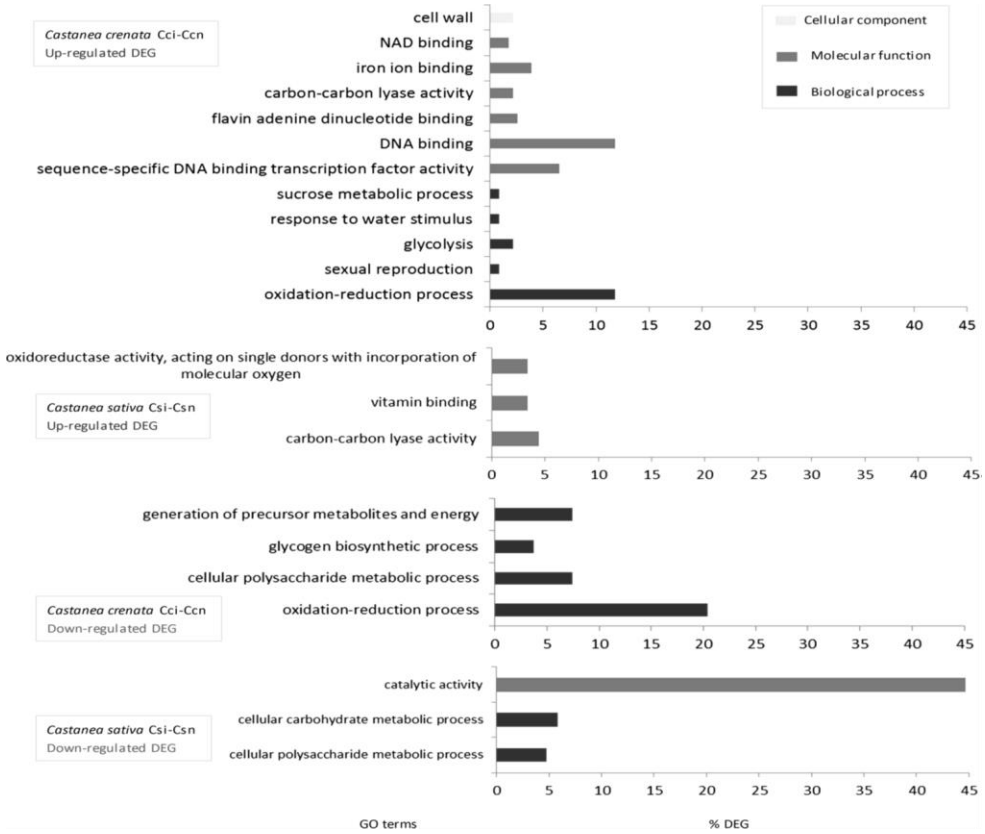


Figure 4. Distribution of *Castanea crenata* and *Castanea sativa* differentially expressed genes (DEGs) into functional sub-categories of Gene Ontology. Enriched Analysis was applied separately to upregulated and downregulated DEGs and compared with the reference sets of all contigs in the comparison, to obtain significant GO terms. The selected *P* value on Fisher's Exact Test was lower than 5E−3.

P. cinnamomi resistance-related genes

We consider that the most significant candidate genes of resistance to *P. cinnamomi* are *C. crenata* (resistant species) genes up-regulated at least 2 times after inoculation that are not present among *C. sativa* DEGs and are putatively related to stress response. A selection of *C. crenata* candidate genes was inferred from the Cci-Ccn DEGs list in Supplementary material 3 and assigned to the functional categories in Table 2.

Table 2 *Castanea crenata* (Japanese, resistant) candidate genes to *Phytophthora cinnamomi* resistance. Genes were associated into the functional categories in the left column (Bold).

Predicted function / Gene	**Reported function, notes**	**Cci/Ccn Log2(fold change)**
Pathogen recognition		
Disease resistance protein At4g27190		2,41
Probable LRR receptor-like serine/threonine-protein kinase	Precursor. Accession: At1g07650	1,81
Probable serine/threonine-protein kinase	Accession At1g54610	1,53
Regulation of host response after pathogen perception		
C2 domain-containing protein	Accession: At1g53590	2,15
Uncharacterized protein At3g61260	Homology with remorins	2,08
Probable WRKY transcription factor 31		1,71
Putative receptor protein kinase ZmPK1	Precursor	1,55
WRKY transcription factor 22	Induction of JA pathway	1,33
GRAS family protein 4	TF	1,20
Calcium-dependent protein kinase isoform 3 CDPK3	SA and phytoalexin synthesis. Induced by JA	1,14
Lipid signaling		
Clathrin interactor EPSIN 3		2,23
IST1-like protein		2,02
Patatin-05	Precursor. Triggering of JA pathway during HR. May be up-regulated by SA and ethylene	1,27
Regulation of plant immune response		
RING finger protein 5	Ubiquitin/proteasome system. JA-dependent signaling	2,97
Putative *syntaxin-24*	Probable SNARE complex-mediated immunity to pathogenic fungi at the plant cell wall	2,58

Predicted function / Gene	**Reported function, notes**	**Cci/Ccn Log2(fold change)**
Sulfate transporter 3,1	Up-regulated in response to pathogens, MeJA and SA. Brassinosteroid hormone regulation	2,25
Ethylene-responsive transcription factor 4	Regulation of JA-responsive defense genes' expression to fungal pathogens	1,50
Ocs element-binding factor 1	TF. Increased activity by SA	1,13
Regulation of drought stress		
NAC domain-containing protein 72	TF	2,66
Myb-related protein 21	TF. Responsive to JA	1,97
Cysteamine dioxygenase	Mediates the response to hypoxia allied to ERF-VII TFs	1,77
Hypersensitive response		
Cationic peroxidase 1	Precursor. Induces cell wall lignification	3,29
Arginine decarboxylase	May be induced by MeJA and SA. Induces polyamine synthesis	1,19
S-adenosylmethionine decarboxylase alpha chain	Precursor. Induces cell wall strengthening and polyamine synthesis	1,05
Hypersensitive response recovery		
Probable glutathione S-transferase	Regulated by SA	2,93
B-cell receptor-associated protein 31	Regulation of apoptosis	1,90
Pto-interacting protein 1	Induced by SA	1,42
Anti-fungal enzymes		
Probable carboxylesterase 120	Serine hydrolase involved in plant-pathogen interactions	5,91
Glucan 1,3-beta-glucosidase	Precursor. Induced by JA. Exported to apoplast	2,67
Acidic endochitinase	Precursor. Induced by SA	2,44
Basic 7S globulin 2	Precursor. May act on pathogen secreted enzymes upon host infection	1,97
Glucan endo-1,3-beta-glucosidase 11	Family 17 glycosyl hydrolases	1,68
Anti-fungal metabolite synthesis		
UDP-glycosyltransferase 85A2 and *85A5*	Flavonoid pathway	3,19-2,49

Predicted function / Gene	**Reported function, notes**	**Cci/Ccn Log2(fold change)**
Flavin-containing monooxygenase FMO GS-OX5	Glucosinolate pathway	2,45
Taxadiene 5-alpha hydroxylase	Taxol (terpene pathway). May be induced by JA	2,42
Taxane 13-alpha-hydroxylase	Taxol (terpene pathway). May be induced by JA	2,06
Squalene monooxygenase	Saponin (terpene pathway). Induced by JA	1,76
L-allo-threonine aldolase	Saponins (terpene pathway) and isoflavonoids (flavonoid pathway). May be induced by JA	1,40
Reticuline oxidase-like protein	Precursor. Alkaloid pathway. Induced by MeJA	1,30
3-ketoacyl-CoA synthase 11	Cuticle. May be induced by SA	1,07
Cell wall strengthening		
Pectinesterase 2	Precursor. Responsive to JA. Acts on homogalacturan	2,98
Pyruvate kinase, cytosolic isozyme	Lignin synthesis upon fungal infection	1,39
Extensin-2	Precursor. Responsive to fungal infection	1,30
6-phosphofructokinase 3	Lignin synthesis	1,26
Cell wall synthesis		
UDP-glucose 4-epimerase GEPI48		2,39
COBRA-like protein 7	Precursor	1,43
UDP-glucose 6-dehydrogenase	Reponsive to pathogen. May be induced by SA	1,20
Response to drought stress		
Phosphoprotein ECPP44	Homology with a dehydrin gene	1,97
Stress recovery		
Probable *arylformamidase*	Alternate pathway for NAD biosynthesis	3,30
Equilibrative nucleoside transporter 2	Nucleotide synthesis rescue pathway	2,67
Protein translation factor SUI1 homolog 2		2,44
Lon protease homolog 2, peroxisomal	Lateral root formation	2,29
Glutamate decarboxylase 1		2,17
Ethanol tolerance protein GEKO1		2,15
Prolyl 4-hydroxylase subunit alpha-2	Precursor. Root hair formation	1,86

Especially noteworthy are the kinase receptor genes that may be involved in pathogen recognition (as the *Probable LRR receptor-like serine/threonine-protein kinase*) (Diévart and Clark 2003) and genes corresponding to TF involved in the regulation of host response after pathogen perception (as *WRKY* TF) (Yang et al. 2009). The putative involvement of JA and salicylic acid (SA) signaling pathways was inferred from the categories 'Regulation of host response after pathogen perception' and 'Lipid signaling'. Two genes in the 'Regulation of plant immune response' category are responsive to the plant hormone ethylene (e.g. *Ocs element-binding factor 1*) (Zhang and Singh 1994).

Certain *C. crenata* up-regulated genes may prevent pathogen progress, such as the precursor of *Cationic peroxidase 1* (Reimers et al. 1992) [category 'Hypersensitive response' (HR)], and *Pectinesterase 2* (Wen et al. 2013) (category 'Cell wall strengthening'). Three genes, e.g. *Probable glutathione S-transferase*, were associated in the category 'HR recovery' (Ryu et al. 2009). Genes involved in 'Anti-fungal metabolite synthesis' (such as *UDP-glycosyltransferase 85A2*) *(Woo et al. 2007)* and 'Anti-fungal enzymes' (such as *Probable carboxylesterase 120*) (Marshall et al. 2003) may take part in the host response to enhance the defense to *P. cinnamomi*.

Genes in the categories 'Regulation of drought stress', 'Response to drought stress' and 'Stress recovery' are also represented in the *C. crenata* candidate list, such as *NAC domain-containing protein 72* (Singh et al. 2013), *Phosphoprotein ECPP44* (Tan and Kamada 2000) and *Lon protease homolog 2 peroxissomal* (Lingard and Bartel 2009), respectively.

C. sativa DEGs after *P. cinnamomi* inoculation are presented in Supplementary material 4. In order to identify the susceptible species' response to the pathogen we selected up-regulated genes in inoculated *C. sativa* (at least two times) that are not present among *C. crenata* DEGs and are putatively related to stress response. Those genes were distributed in a series of functional categories in Table 3. In the 'Regulation of plant immune

response' category we emphasize the gene *REF/SRPP-like protein At1g67360* (Taki et al. 2005), induced by a precursor of JA. *C. sativa* also invests in genes related to 'Stress recovery' (e.g. *Aminophospholipid flippase 9*) (López-Marqués et al. 2012) and 'HR recovery' (e.g. *4-hydroxyphenylpyruvate dioxygenase*) (Peal et al. 2011). Genes involved in 'Anti-fungal metabolite synthesis' (e.g. *Flavonoid 3-hydroxylase*) (Sharma et al. 2012) and 'Cell wall strengthening' (e.g. *UPF0497 membrane protein At3g06390*) (Roppolo et al. 2011) may prevent *P. cinnamomi* proliferation in the host. Finally, *C. sativa* up-regulated genes linked to drought stress regulation, such as the TF *Homeobox-leucine zipper protein HAT5* (Henriksson et al. 2005) may play a role in host recovery from pathogenicity.

Validation of RNA-seq

The RNA-seq approach allowed for the quantification of gene expression levels by sequence read depth. DEGs were identified by estimating the ratio between reads in inoculated libraries and non-inoculated controls (Cci-Ccn, Csi-Csn). To validate the differential expression levels observed by RNA-seq, qRT-PCR was used to obtain the expression level of DEGs in inoculated libraries (Cci, Csi) relative to non-inoculated libraries (Ccn, Csn). The selected DEGs (Figure 5) are putatively related to *Castanea* response to the pathogen and to host recovery, and include: *Ethylene-responsive TF 4*, *Disease resistance protein At4g27190*, *Ethylene-responsive TF ABR1*, *Precursor of glucan 1,3-beta-glucosidase* (family 5), *Pectinesterase 2* and *C2 domain-containing protein At1g53590*.

The differential gene expression for the comparisons Cci-Ccn and Csi-Csn acquired with the 454 sequencing was compared with the relative expression levels obtained with qRT-PCR for the selected DEGs.

Table 3 *Castanea sativa* (European, susceptible) up-regulated genes upon *Phytophthora cinnamomi* inoculation related to stress response. Genes were associated into the functional categories in the left column (Bold).

Predicted function / Gene	**Reported function, notes**	**Csi/Csn Log2(fold change)**
Regulation of host response after pathogen perception		
Serine/threonine-protein phosphatase PP1 isozyme 4		1,90
SNF1-related protein kinase regulatory subunit γ 1	May interact with LRR-rich proteins	1,44
Regulation of plant immune response		
DEAD-box ATP-dependent RNA helicase 56		1,57
REF/SRPP-like protein At1g67360	Induced by the precursor of JA pathway OPDA	1,28
Histone deacetylase 6	Interacts with Ethylene and JA pathways related with plant defense to necrotrophic pathogens	1,28
Regulation of drought stress		
Homeobox-leucine zipper protein HAT5	TF. Responsive to ABA and water deficit stress	2,27
Zinc finger A20 and AN1 domain-containing stress-associated protein 11	May act in protein targeting to the ubiquitin-proteasome pathway	1,44
Myb-related protein 44	TF. Modulates SA and JA signaling	1,30

Predicted function / Gene	**Reported function, notes**	**Csi/Csn Log2(fold change)**
Ethylene-responsive transcription factor RAP2-4	Induced by JA. Related to alkaloid metabolism control	1,01
Hypersensitive response		
Autophagy-related protein 9		2,44
Hypersensitive response recovery		
RNA-binding post-transcriptional regulator csx1	Regulatory protein, pathogen-responsive, acting during oxidative signaling	1,75
4-hydroxyphenylpyruvate dioxygenase	Plastoquinone synthesis. Up-regulated by MeJA	1,49
Phosphoserine aminotransferase, chloroplastic	Precursor. JA responsive gene. Pyridoxal synthesis	1,38
Late embryogenesis abundant protein Lea5		1,22
Anti-fungal enzymes		
Oxalyl-CoA decarboxylase	Oxalate catabolism	2,96
Protein TAR1	Inhibition of fungal laccase	1,67
Anti-fungal metabolite synthesis		
Flavonoid 3-hydroxylase	Flavonoid pathway	2,34
Uncharacterized membrane protein At4g09580	Homology with SNARE associated Golgi protein. Probable secretion of antimicrobial molecules and cell wall components into the apoplast	1,18
Cell wall strengthening		

Predicted function / Gene	**Reported function, notes**	**Csi/Csn Log2(fold change)**
UPF0497 membrane protein At3g06390	Casparian strip membrane proteins' family	2,11
Alcohol dehydrogenase	Probable cinnamyl-alcohol dehydrogenase involved in lignin synthesis	1,11
Cell wall synthesis		
Probable galacturonosyltransferase-like 9	May be involved in pectin and/or xylan biosynthesis	1,55
Stress recovery		
Aminophospholipid flippase 9	Establishment of phospholipid asymmetry in plasma membrane	4,35
Universal stress protein MJ0531		2,21
Auxin-repressed 12.5 kDa protein	Probably related to drought stress recovery	1,11
E3 ubiquitin-protein ligase BRE1-like 2	Involved in root growth regulation through histone ubiquitination	1,05
High affinity cationic amino acid transporter 1	Drought stress recovery. Induced by JA and ABA	1,03

The results presented in Figure 5 reveal differences in the expression levels of *C. crenata* transcripts upon inoculation when compared to *C. sativa* transcripts upon inoculation. Those differences are in accordance with read data obtained by RNA-seq and may reflect *Castanea* root transcriptome in response to *P. cinnamomi*.

Discussion

Functional annotation

GO annotation comparison of expressed genes after inoculation between Japanese (*C. crenata*, resistant to pathogen) and European chestnut (*C. sativa*, susceptible to pathogen) revealed a correlation of gene ontology, suggesting a convergent response after pathogen inoculation. However, among DEGs, GO annotation revealed differences that suggest distinct host susceptibility to the pathogen as well as variations in gene expression and timing. *C. crenata* inoculated with the pathogen up-regulated genes with the functional GO annotation 'Oxidation reduction process' (BP), disclosing genes involved in the synthesis of anti-fungal secondary metabolites (6 in 27) and in stress recovery (10 in 27). Examples are *Squalene monooxygenase* (Belchí-Navarro et al. 2013) and *Prolyl 4-hydroxylase subunit alpha-2* (Vlad et al. 2007), respectively. On other hand, 'Sequence-specific DNA binding transcription factor activity' (MF) point to genes coding for TF related to pathogen recognition and biotic stress regulation (10 in 14, examples in Table 2).

Contrasting with *C. crenata*, GO annotation for inoculated *C. sativa* revealed that the down-regulated genes in 'Catalytic activity' (MF) were involved in the synthesis of secondary metabolites, protein kinases and receptor-like protein kinases (24 in 84). In the same term are also included genes related to stress recovery (9 in 84).

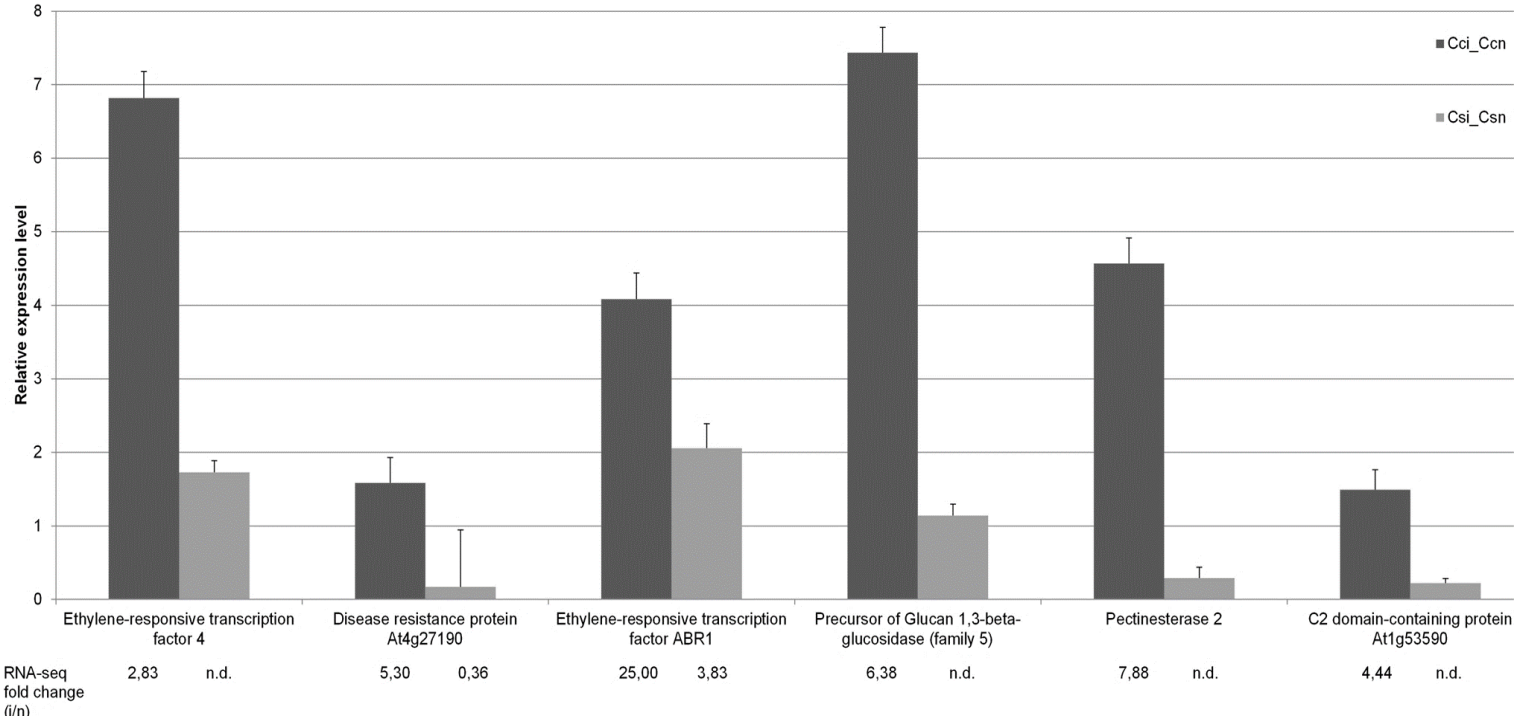


Figure 5. Relative expression of DEGs by quantitative real-time PCR and comparison with 454 sequencing read ratio. Transcript quantitative expression was normalized with the reference gene *actin-7* in the pools Cci (inoculated Japanese), Ccn (non-inoculated Japanese), Csi (inoculated European) and Csn (non-inoculated European). Cci was calibrated against Ccn and Csi was calibrated against Csn to determine relative expression level, following the ΔΔCT method described in Material and Methods. Error bars correspond to the standard deviation of the mean of three technical replicates. Fold change between inoculated libraries and non-inoculated libraries (i/n, *P* value<1E-3) is shown below the chart for the respective gene and species. n.d.: non-detected.

Inoculated *C. sativa* up-regulated genes are significantly annotated to 'Carbon-carbon lyase activity' (MF) and almost all genes are involved in glycolysis (4 in 5). The predominance of down-regulated genes in *C. sativa* upon pathogen inoculation is in accordance with the species' higher susceptibility to *P. cinnamomi*. A review by Oßwald et al (Oßwald et al. 2014) states that the reactions of susceptible woody plant hosts to *Phytophthora* spp. include a broad down-regulation of defense-related genes in infected roots, promoting pathogen growth.

Pathogen response in resistant Japanese chestnut

Upon infection, Japanese chestnut up-regulated twice the number of DEGs when compared with the susceptible European chestnut. Among these, we selected a set of Japanese chestnut candidate resistance genes, taking into consideration the regulation (all up-regulated), the best hit description (Interpro and BLAST), the protein function and the relevance to pathogen defense and host recovery. Candidate genes included those putatively involved in pathogen recognition, such as *LRR receptor-like serine/threonine-protein kinase At1g07650,* coding for a transmembrane protein involved in the recognition of pathogen elicitor complexes that locally activate defense-related pathways (Diévart and Clark 2003). Japanese chestnut also up-regulated TF which are reportedly active early in the elicitor-induced defense response (e.g. *WRKY transcription factors 22* and *31*) (Zhang et al. 2008b; Yang et al. 2009). One Japanese chestnut up-regulated gene involved in lipid signaling corresponds to *Patatin-05*, a Phospholipase A2-related gene. This gene shows a rapid transcriptional activation in virus-infected leaves preceding the increase of Phospholipase A2, which may provide precursors for the synthesis of the JA class of oxylipins during HR (Dhondt et al. 2000).

Japanese chestnut genes which are up-regulated upon pathogen inoculation and are involved in plant immune response regulation include *RING finger*

protein 5, which may contribute to elicitor-activated response via a JA-dependent signaling pathway (Hondo et al. 2007).

Recognition of a pathogen often triggers a localized resistance reaction known as the hypersensitive response (HR), which is characterized by the production of reactive oxygen species (ROS), leading to accelerated cell death and inhibition of pathogen spread (Hammond-Kosack and Jones 1997). Six Japanese chestnut candidate genes for *P. cinnamomi* resistance may be involved in HR. Cationic peroxidase 1 induces cell wall reinforcement through lignin deposition (Reimers et al. 1992) and is potentially involved in the defense of *Quercus suber* and avocado to *P. cinnamomi* (Coelho et al. 2011; Reeksting et al. 2014). *Probable glutathione S-transferase* may be related with the reduction of glutathione and consequent protection against the adverse effects of oxidative reactions, such as membrane lipid peroxidation during pathogen infection (Ryu et al. 2009). Rookes et al (2008) observed an elevation of *Glutathione S-transferase 1* expression in *P. cinnamomi*-inoculated roots of *A. thaliana* Col-0, an ecotype which is considered tolerant to the pathogen.

Most of the Japanese chestnut candidate genes classified in the category 'Anti-fungal enzymes' (Table 2) code for glycoside hydrolases. For example, *Acidic endochitinase* was reported to be a putative defense-related gene in the response of avocado to *P. cinnamomi* (Reeksting et al. 2014).

Eight Japanese chestnut candidate genes may be related to anti-fungal metabolite synthesis. Specifically, *UDP-glycosyltransferase 85A2* and *85A5* and *L-allo-threonine aldolase* may act in flavonoid synthesis (Schopfer and Ebel 1998; Broeckling et al. 2005; Woo et al. 2007). Flavonoids are synthesized by the phenylpropanoid pathway. Many phenylpropanoid compounds are stress-induced, comprising physical and chemical barriers against pathogen infection (such as lignin and suberin) and signal molecules involved in local and systemic signaling for defense gene induction (Dixon and Paiva 1995). Four candidate genes related to anti-fungal metabolite

synthesis are induced by the JA pathway: *Taxane 13-α-hydroxylase* (involved in taxol synthesis, an Oomycete defense compound in *Tax*us) (Sun et al. 2013), *Squalene monooxygenase* (Hu et al. 2003), a precursor of *Reticuline oxidase-like* protein (Shoji and Hashimoto 2011; Belchí-Navarro et al. 2013) and the above mentioned *L-allo-threonine aldolase*.

Plant disease resistance depends partially on the host's ability to restrict pathogen development at the cell-surface level (Cantu et al. 2008). Structural changes in the host cell wall related to defense mechanisms include deposition of lignin-like material, production of callose and phenolic compounds, and accumulation of hydroxyproline-rich glycoproteins (Benhamou et al. 1991).

Four Japanese chestnut candidate genes were associated in the 'Cell wall strengthening' category (Table 2). Two genes encode glycolysis enzymes that may be involved in cell wall lignification: cytosolic *Pyruvate kinase* and *6-fosphofructokinase 3*. Mutuku and Nose (2012) reported that those enzymes were highly expressed in *Rhizoctonia solani*-infected rice plants and suggested that the regulation of glycolysis in those conditions was involved in carbon allocation for other pathways such as the phenylpropanoid pathway for lignin synthesis. *Extensin-2* encodes a hydroxyprolin-rich glycoprotein that increases in resistant cultivars of tomato infected by *Fusarium oxysporum* (Benhamou et al. 1991).

Since *P. cinnamomi* infection results in drought stress symptoms, we identified four Japanese chestnut candidate genes involved in the response to drought stress (Table 2). One of them, *Phosphoprotein ECPP44,* encodes a protein that specifically protects cells from desiccation damage (Tan and Kamada 2000).

After pathogen attack and host response towards disease tolerance or resistance, plants then attempt to return to a normal status. *C. crenata* up-regulated seven genes classified in the functional category 'Stress recovery'. *Equilibrative nucleoside transporter 2*, for example, is related to a rescue

pathway of nucleotide synthesis (Li et al. 2003). Root damage by *P. cinnamomi* probably resulted in the up-regulation of three genes associated with the root system, including *Lon protease homolog 2* and *Prolyl 4-hydroxylase subunit alpha-2*, involved in root formation (Vlad et al. 2007; Lingard and Bartel 2009).

Pathogen response in susceptible European chestnut

Upon inoculation, about half the number of DEGs were up-regulated in the European chestnut plants when compared to Japanese chestnut, while about two thirds as many DEGs were down-regulated. After analysis of the best hit description (Interpro and BLAST), European chestnut DEGs putatively linked to a pathogen response and host recovery were associated to the functional categories shown in Table 3. Examples include *Serine/threonine-protein phosphatase PP1 isozyme 4*, which codes for a phosphatase that may regulate signal transduction initiated by pathogen elicitors (Lin et al. 1999), and *Histone deacetylase 6*, which codes a for a protein that interacts with enzymes of the ET and JA pathways related to plant defense to necrotrophic pathogens (Zhu et al. 2011).

Fungal pathogens can use oxalate as a phytotoxin to promote plant infection. European chestnut significantly up-regulated *Oxalyl-CoA decarboxylase* upon inoculation that may take part of an oxalate catabolism pathway (Foster et al. 2012). Transgenic potato and taro plants transformed with a wheat oxalate oxidase gene demonstrated increased resistance to the Oomycete pathogens *P. infestans* and *P. colocasiae* respectively (Schneider et al. 2002; He et al. 2013), which comes in accordance with the putative role of the oxalate catabolism in the host defense to *Phytophthora*. The American chestnut restoration program is partially based on genetic transformation with the same oxalate oxidase gene, so the host can degrade the oxalate secreted by the fungus *C. parasitica* during infection (Zhang et al. 2013). The *Protein TAR1* gene up-regulated in inoculated European chestnut is reported

to be involved in fungal laccase inhibition upon host infection (Jiang et al. 2009). Fungi can adopt Cu-containing polyphenol oxidases or laccases to degrade plant lignin during infection. Feng and Li (Feng and Li 2012) identified laccase genes in *Phytophthora* spp., providing the hypothesis of a similar role of laccases in Oomycete pathogenicity.

Five up-regulated European chestnut genes were associated in the 'Stress recovery category'. Two of them are likely related to drought stress recovery, such as the *High affinity cationic amino acid transporter 1* (*CAT1*). According to Liu and Bush (Liu and Bush 2006), the promoter of the homologous gene *AtCAT1* has motifs responsive to methyl JA and drought. In birch, Cu-induced oxidative stress may induce the CAT1-like transporter, which then delivers amino acids used in cellular repair processes (Keinänen et al. 2007). When the set of European chestnut genes down-regulated upon inoculation was analysed in terms of best hit description (DEGs in Supplementary material 4, Interpro and BLAST columns), several of them were found to correspond to up-regulated genes in inoculated *C. crenata* (DEGs in Supplementary material 3). This suggests that infection of European chestnut plants by *P. cinnamomi* had a significant influence on host gene regulation, resulting in damage to the host. For example, *Myb-related protein 306* corresponds to an activator of anthocyanin synthesis genes (Jackson et al. 1991) and its inhibition could constrain the synthesis of some anti-fungal metabolites. The down-regulation of *Peroxisomal-coenzyme A synthetase* may repress the JA pathway activation (Schneider et al. 2005). Repression of *Expansin-like A1* may affect cell wall synthesis (Irshad et al. 2008) and therefore may contribute to the spread of the pathogen in the host. Down-regulation of *Pleiotropic drug resistance protein 2*, an ABC transporter of plant secondary metabolites (Yazaki 2006), probably prevents anti-fungal metabolites from reaching the pathogen. Repression of *Probable methyltransferase PMT2* may reduce synthesis of nicotine (Shoji and Hashimoto 2011), which could otherwise act as a fungicide. *Glutamate*

decarboxylase 1 codes for root-specific calcium/calmodulin-regulated GAD1, which plays a major role in GABA synthesis in plants responding to stress, thereby helping maintain plant homeostasis (Bouché and Fromm 2004). Thus the repression of *GAD1* may affect host recovery from pathogen attack. Glutaredoxins are candidates for mediating redox regulation of transcriptional regulators that target genes associated with detoxification and pathogen defense (Ndamukong et al. 2007). The *Glutaredoxin-C9* gene was highly up-regulated in Japanese chestnut; its down-regulation in European chestnut may also affect host recovery.

Comparison between Japanese and European chestnut response

DEGs annotation analyses revealed that Japanese and European chestnuts show many common features in their responses to *P. cinnamomi*. However, this evaluation must be carefully regarded as it is not supported by transcript profiling or functional analysis. Upon *P. cinnamomi* inoculation, both species up-regulated genes involved in HR/HR recovery, genes related to the regulation of JA pathway and genes induced by JA related to anti-fungal metabolite synthesis and anti-fungal enzymes. The presence of HR and JA signaling upon pathogen inoculation indicates that both host species were able to recognize the pathogen attack. According to Thomma (Thomma 1998), the JA-dependent defense response pathway is required for resistance to necrotrophic pathogens. Eshragui et al (Eshraghi et al. 2014) suggest that a *P. cinnamomi* challenge activates JA-related plant defense responses in leaves of *A. thaliana* Col-0. HR is suggested to be associated with all forms of resistance to *Phytophthora* (Kamoun et al. 1999) and is believed to constitute one of the primary mechanisms of resistance to plant pathogens. Induction of HR is often associated with synthesis of antimicrobial compounds and cell wall thickening (Hammond-Kosack and Jones 1996). We identified *C. crenata* and *C. sativa* DEGs related to cell wall strengthening and anti-fungal metabolite synthesis. HR also induces several genes

involved in cellular protection (Jabs et al. 1996). We also identified DEGs related to HR recovery in both species. The suggested occurrence of HR in inoculated European chestnut, the susceptible species, potentially points to partial resistance within this genotype. Partial resistance to *Phytophthora infestans* is common in wild *Solanum* species, which may reveal HR-like necrotic reactions and, occasionally, late or trailing HR. This suggests a weak R gene-Avr gene interaction or a gene-dosage effect resulting in ineffective HR and partly resistant phenotypes (Kamoun et al. 1999).

Japanese chestnut resistance to ink disease may in part result from a set of up-regulated genes during *P. cinnamomi* attack involved in pathogen recognition, regulation of host response after pathogen perception, and signaling through lipids. When compared to Japanese chestnut, European chestnut up-regulated much less genes in those functional categories. Current knowledge describes the plant immune response as starting with the recognition of pathogen elicitors by plant receptors, followed by induction of resistance genes (R genes) that initiate signal transduction cascades leading to: a) HR and rapid cell death and b) the activation of phytohormone signaling pathways [reviewed in (Bari and Jones 2009)]. In our study inoculated Japanese chestnut induced DEGs involved in the SA pathway regulation (e.g. *Calcium-dependent protein kinase isoform 3*) (Chung et al. 2004) as well as DEGs induced by SA related to the regulation of plant immune response (e.g. *Sulfate transporter 3,1*) (Marsolais et al. 2007) and HR (e.g. *Arginine decarboxylase*) (Nakane et al. 2003). García-Pineda et al (2009) observed that SA inhibited avocado root colonization in the interaction between *Persea americana* and *P. cinnamomi*. In the complex web of defense responses JA, SA, Ethylene and Abscisic Acid are essential players (Bari and Jones 2009). SA is activated during and following HR (Jabs et al. 1996) and is generally involved in the activation of defense responses against biotrophic and hemi-biotrophic pathogens, as well as in the establishment of systemic acquired resistance (Bari and Jones 2009).

Vleeshouwers et al (Vleeshouwers et al. 2000) studied the *P. infestans*-*Solanum* interaction using wild species and reported that in fully resistant genotypes, the HR was faster and resulted in smaller lesions than in partially resistant clones. The authors suggest that the difference between compatibility (non-resistant host response) and incompatibility (resistant host response) is quantitative rather that qualitative. In our study, Japanese chestnut regulated a higher number of genes involved in biotic stress upon *P. cinnamomi* inoculation when compared to the European chestnut. The identified DEGs are not only related to HR but also with cell wall strengthening, anti-fungal metabolite synthesis and anti-fungal enzyme synthesis, and may account for the Japanese chestnut's adequate resistance to ink disease.

Castanea response to *P. cinnamomi* and *C. parasitica*: brief comparison

The reports of Barakat et al. (2009, 2012) provided the first insights into chestnut resistance to *C. parasitica* using high-throughput RNA-seq. The response of chestnut to *C. parasitica* and *P. cinnamomi* may be comparable, as fungi and Oomycetes share similar infection mechanisms (Latijnhouwers et al. 2003). When comparing Chinese and American chestnut responses to *C. parasitica* with the Japanese and European chestnut responses to *P. cinnamomi*, we found similar DEGs that fall in the following functional categories: a) Regulation of biotic stress response (*ATPase transporter*, *Pyridine nucleotide-disulphide oxidoreductase*), b) HR and cell wall lignification (*Peroxidase*), c) HR recovery (*Arginine decarboxylase*, *Manganese superoxide dismutase*), d) Anti-fungal enzymes (*Thaumatin-like protein*, *β-1,3-glucanase*, *Chitinase*), e) Anti-fungal metabolite synthesis (family 1 *Cytochrome P450 glycosyltransferase*, *Abscisic acid 8'-hydroxylase*, *Squalene monooxygenase*, *UDP-glucosyltransferase*), f) Cell wall synthesis (*β-expansin*), and g) Stress recovery (*ABC transporter family*, *Glyceraldehyde 3-phosphate dehydrogenase*). Other shared *Castanea*

responses to both pathogens include DEGs related to kinase genes involved in pathogen recognition and JA pathway activation, gene regulation by Myb TF and Ethylene-responsive TF, and genes of the 26S proteasome regulatory unit. The response of all four species to both pathogens further includes genes from the flavonoid pathway that promote phytoalexin synthesis.

In summary, the DEG analysis of *C. sativa* and *C. crenata* root transcriptomes after *P. cinnamomi* inoculation revealed similarities among the four *Castanea* species response to both pathogens, namely genes related to systemic acquired resistance, HR that may prevent pathogen spread and the putative involvement of JA pathway. Some of these DEGs may also promote cell wall strengthening through lignification and synthesis of flavonoids as anti-fungal metabolites.

Final considerations

RNA-seq using 454 platform was adequate for comparing the root transcriptomes of two Fagaceae species, *Castanea sativa* and *Castanea crenata* when either inoculated or non-inoculated with the pathogen *Phytophthora cinnamomi*. The four sequenced transcript libraries allowed a draft comparison of both species' responses to the pathogen in terms of gene regulation and pathways, together with the selection of candidate genes for host resistance to *P. cinnamomi*. Although further research is required on gene expression at specific time points after inoculation, *in silico* analysis has shown that Japanese and European chestnut, despite the association of expressed genes in similar functional categories, differ in the distribution of DEGs after pathogen inoculation. The most noteworthy result from DEG analysis was the overall down-regulation of genes in susceptible *C. sativa*, which may facilitate the pathogenicity of *P. cinnamomi*. On the other hand, in the resistant *C. crenata* there was the regulation of a higher number of genes related with biotic stress when compared to *C. sativa*, mostly up-

regulated. Analysis of homology and functional annotation revealed associations between many of those up-regulated genes with pathogen response in other plant species, and suggests involvement in pathogen recognition, regulation of the host immune response, signaling, hypersensitive response, cell wall strengthening and encoding of enzymes and synthesis of metabolites against Oomycetes and fungal pathogens. The regulation of DEGs in *C. crenata* and many of the specific transcripts we identified may account for the adequate resistance level of this species to *P. cinnamomi*.

SSR markers were also developed from the sequences of these candidate genes in order to improve the mapping approach for identification of QTLs related to pathogen resistance in Japanese and European chestnut (Costa et al. 2011 and Chapter V). In Chapter V we will analyse if the candidate genes map to disease resistance QTLs, which will provide further support for a major role in chestnut resistance to the pathogen.

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Data Archiving Statement

Raw data files can be accessed in the Short Read Archive at NCBI (http://www.ncbi.nlm.nih.gov) with the reference PRJNA215368. Nucleotide

and aminoacid sequences are publicly available in the Fagaceae Genomics Web (http://www.fagaceae.org/).

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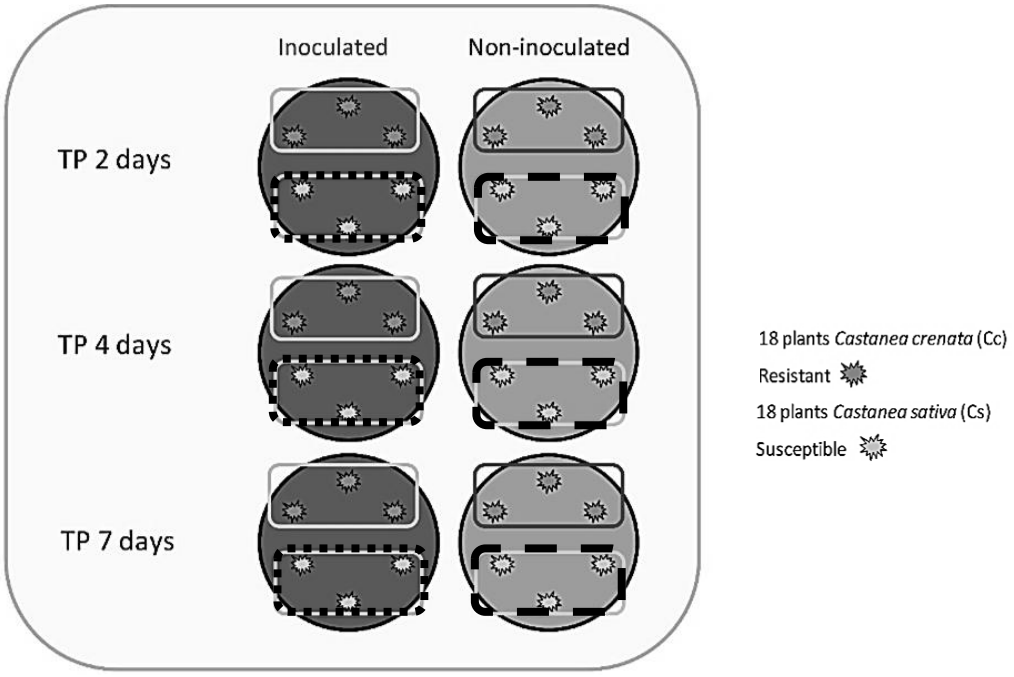
Supplementary material

Supplementary material 1. Experimental design and plant material. Circles represent soil pots. Rectangles represent the sample collection for the pools: Grey line: Cci; Black line: Ccn; Rond dots: Csi and Dash line: Csn. TP: time point.



Supplementary material 2. Primers used in quantitative real-time PCR for RNA-seq validation.

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
Actin-7	CCAAGGCCAACAGGGAAAA	CGGCCTGGATAGCAACATACA
Ethylene-responsive transcription factor 4	CCCTCCTGATTCTGCTTCT	TTGGTGTTGTGGTGCTTGT
Disease resistance protein At4g27190	AAGGGAGAGGAAGACGGA	TGTGGGTGGGGAAAATGAA
Ethylene-responsive transcription factor ABR1	GGGCGTGAAGAAGAAGAAG	GTGGTGGTGGTGGTGGTT
Precursor of Glucan 1,3-beta-glucosidase	GGGTTACAGGGCGAGTTT	CGTTGGTTTGCTTGCTATC
Pectinesterase 2	TGAAAAGGCTGCATGGGCT	GGTTTGGCTGCACTTGACA
C2 domain-containing protein At1g53590	TGTCAACCCAGCCAAGTTTT	CCCGAGTACATGCGAACAAC

Supplementary material 3, **4** and **5** can be found at http://link.springer.com/article/10.1007/s11295-014-0829-7, section 'Supplemetary material'. File names: 11295_2014_829_MOESM3_ESM.xlsx, 11295_2014_829_MOESM4_ESM.xlsx and 11295_2014_829_MOESM6_ESM.xlsx, respectively.

Supplementary material 3 Differentially expressed genes in *Castanea crenata* after *Phytophthora cinnamomi* inoculation (Cci) versus non-inoculated library (Ccn), *P* value<1E-3.

Supplementary material 4 Differentially expressed genes in *Castanea sativa* after *Phytophthora cinnamomi* inoculation (Csi) versus non-inoculated library (Csn), P value<1E-3.

Supplementary material 5 Differentially expressed genes in *Castanea crenata* and *Castanea sativa* after *Phytophthora cinnamomi* inoculation (Cci-Csi), *P* value<1E-3.

Supplementary material 6 Differentially expressed genes in *Castanea sativa* non-inoculated (Csn) versus *Castanea crenata* non-inoculated library (Ccn), *P* value<1E-3. Tables are presented from page 121 to 137.

Contig name	Nº a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_010311	407	525	249	3,56E-17	2,11	1,08	IPR000873	AMP-dependent synthetase/ligase	AMP dependent ligase, putative [Ricinus communis]
CCN_CSN_007647	160	11	82	3,80E-05	0,13	-2,90	IPR000266	Ribosomal protein S17	RecName: Full=40S ribosomal protein S11-1; AltName: Full=Protein EMBRYO DEFECTIVE 1080
CCN_CSN_001440	432	8	80	4,02E-05	0,10	-3,32	IPR012919	Sad1/UNC-like, C-terminal	RecName: Full=SUN domain-containing protein 2; AltName: Full=Protein unc-84 homolog B; AltName: Full=Rab5-interacting protein; Short=Rab5IP; AltName: Full=Sad1/unc-84 protein-like 2
CCN_CSN_002624	339	145	53	4,24E-05	2,74	1,45	IPR002067	Mitochondrial carrier protein	RecName: Full=Probable mitochondrial 2-oxoglutarate/malate carrier protein; Short=OGCP; AltName: Full=Mitochondrial substrate carrier family protein ucpC; AltName: Full=Solute carrier family 25 member 11 homolog
CCN_CSN_001278	451	651	443	4,25E-05	1,47	0,56	IPR000941	Enolase	RecName: Full=Enolase; AltName: Full=2-phospho-D-glycerate hydro-lyase; AltName: Full=2-phosphoglycerate dehydratase; AltName: Full=OSE1
CCN_CSN_000541	588	389	233	6,51E-05	1,67	0,74	IPR002202	Hydroxymethylglutaryl-CoA reductase, class I/II	RecName: Full=3-hydroxy-3-methylglutaryl-coenzyme A reductase 1; Short=HMG-CoA reductase 1
CCN_CSN_001629	415	8	78	6,64E-05	0,10	-3,29	IPR003121	SWIB/MDM2 domain	RecName: Full=SWI/SNF complex component SNF12 homolog
CCN_CSN_008820	128	167	69	7,56E-05	2,42	1,28	IPR000626	Ubiquitin	RecName: Full=Ubiquitin-60S ribosomal protein L40-2; AltName: Full=Protein EARLY-RESPONSIVE TO DEHYDRATION 16; AltName: Full=Protein EMBRYO DEFECTIVE 2167; AltName: Full=Protein HAPLESS 4; Contains: RecName: Full=Ubiquitin; Contains: RecName:

Contig name	Nº a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
									Full=60S ribosomal protein L40-2; AltName: Full=CEP52; Flags: Precursor
CCN_CSN_000680	551	6	34	0,000101	0,18	-2,50	IPR001841	Zinc finger, RING-type	RecName: Full=E3 ubiquitin-protein ligase synoviolin; AltName: Full=Synovial apoptosis inhibitor 1; Flags: Precursor
CCN_CSN_008499	139	8	37	0,000102	0,22	-2,21	IPR002220	Dihydrodipicolinate synthetase-like	RecName: Full=Dihydrodipicolinate synthase, chloroplastic; Short=DHDPS; Flags: Precursor
CCN_CSN_006952	175	120	66	0,000104	1,82	0,86	IPR000873	AMP-dependent synthetase/ligase	RecName: Full=2-succinylbenzoate--CoA ligase; AltName: Full=OSB-CoA synthetase; AltName: Full=o-succinylbenzoyl-CoA synthetase
CCN_CSN_000124	844	41	86	0,000106	0,48	-1,07	IPR003440	Glycosyl transferase, family 48	RecName: Full=Callose synthase 1; AltName: Full=1,3-beta-glucan synthase; AltName: Full=Protein GLUCAN SYNTHASE-LIKE 6
CCN_CSN_003505	291	54	19	0,000107	2,84	1,51	IPR012317	Poly(ADP-ribose) polymerase, catalytic domain	RecName: Full=Poly [ADP-ribose] polymerase 15; Short=PARP-15; AltName: Full=B-aggressive lymphoma protein 3
CCN_CSN_006823	179	30	2	0,000112	15,00	3,91	IPR003385	Glycoside hydrolase, family 77	RecName: Full=4-alpha-glucanotransferase DPE1, chloroplastic/amyloplastic; AltName: Full=Amylomaltase; AltName: Full=Disproportionating enzyme; Short=D-enzyme; AltName: Full=Protein DISPROPORTIONATING ENZYME 1; Flags: Precursor
CCN_CSN_008081	151	2	30	0,000112	0,07	-3,91	IPR011010	DNA breaking-rejoining enzyme, catalytic core	RecName: Full=DNA topoisomerase 1; AltName: Full=DNA topoisomerase I
CCN_CSN_005371	221	210	100	1,14E-04	2,10	1,07	IPR007087	Zinc finger, C2H2-type	RecName: Full=Zinc finger protein 1; AltName: Full=WZF1
CCN_CSN_007885	155	103	167	0,000121	0,62	-0,70			RecName: Full=Major latex allergen Hev b 5; AltName: Allergen=Hev b 5
CCN_CSN_002133	374	16	49	0,000122	0,33	-1,61	IPR010714	Coatomer, alpha subunit, C-terminal	RecName: Full=Coatomer subunit alpha-1; AltName: Full=Alpha-coat protein 1; Short=Alpha-COP 1
CCN_CSN_006572	186	16	49	0,000122	0,33	-1,61	IPR000566	Lipocalin/cytosolic fatty-acid	RecName: Full=Outer membrane lipoprotein blc; Flags: Precursor

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
								binding protein domain	
CCN_CSN_006091	200	32	5	0,000122	6,40	2,68	IPR004864	Late embryogenesis abundant protein, group 2	RecName: Full=Protein NDR1; AltName: Full=Non-race specific disease resistance protein 1; Short=AtNDR1; Flags: Precursor
CCN_CSN_004440	252	52	18	0,000125	2,89	1,53	IPR000308	14-3-3 protein	RecName: Full=14-3-3 protein 10
CCN_CSN_002417	354	6	33	0,00014	0,18	-2,46	IPR005814	Aminotransferase class-III	RecName: Full=Uncharacterized aminotransferase y4uB
CCN_CSN_000557	585	47	15	0,000141	3,13	1,65	IPR003952	Fumarate reductase/succinate dehydrogenase, FAD-binding site	RecName: Full=Succinate dehydrogenase [ubiquinone] flavoprotein subunit 1, mitochondrial; AltName: Full=Flavoprotein subunit 1 of complex II; Short=FP; Flags: Precursor
CCN_CSN_003516	291	2	29	0,000143	0,07	-3,86	IPR001107	Band 7 protein	RecName: Full=Hypersensitive-induced response protein 4; Short=AtHIR4
CCN_CSN_000012	1099	59	23	0,000148	2,57	1,36	IPR000403	Phosphatidylinositol 3-/4-kinase, catalytic	RecName: Full=Uncharacterized PI3/PI4-kinase family protein C1F5.11c
CCN_CSN_005337	222	23	59	0,000148	0,39	-1,36	IPR001929	Germin	RecName: Full=Germin-like protein subfamily 1 member 16; Flags: Precursor
CCN_CSN_002827	327	202	132	0,00015	1,53	0,61	IPR001471	Pathogenesis-related transcriptional factor/ERF, DNA-binding	RecName: Full=Ethylene-responsive transcription factor 1; AltName: Full=Ethylene-responsive element-binding factor 1; Short=EREBP-1; Short=OsEREBP1
CCN_CSN_007742	158	47	93	0,000152	0,51	-0,98	IPR004827	Basic-leucine zipper (bZIP) transcription factor	RecName: Full=Ocs element-binding factor 1; Short=OCSBF-1
CCN_CSN_002679	336	128	197	0,000153	0,65	-0,62	IPR003657	DNA-binding WRKY	RecName: Full=Probable WRKY transcription factor 17; AltName: Full=WRKY DNA-binding protein 17
CCN_CSN_005314	223	29	3	0,000153	9,67	3,27	IPR000719	Protein kinase, catalytic domain	RecName: Full=Probable receptor-like protein kinase At2g21480; Flags: Precursor

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_008038	152	1	44	0,000166	0,02	-5,46	IPR023214	HAD-like domain	RecName: Full=Magnesium-dependent phosphatase 1; Short=MDP-1
CCN_CSN_009602	84	1	44	0,000166	0,02	-5,46	IPR003749	ThiamineS	RecName: Full=Molybdopterin synthase sulfur carrier subunit; AltName: Full=Molybdenum cofactor synthesis protein 2 small subunit; AltName: Full=Molybdenum cofactor synthesis protein 2A; Short=MOCS2A; AltName: Full=Sulfur carrier protein MOCS2A
CCN_CSN_008555	137	209	294	0,000167	0,71	-0,49	IPR000164	Histone H3	RecName: Full=Histone H3.3; AltName: Full=Histone H3.2
CCN_CSN_000210	747	63	26	0,000169	2,42	1,28	IPR000719	Protein kinase, catalytic domain	RecName: Full=Receptor-like protein kinase HAIKU2; Flags: Precursor
CCN_CSN_010199	539	251	173	0,000172	1,45	0,54	IPR004170	WWE domain	predicted protein [Populus trichocarpa]
CCN_CSN_003006	317	102	54	0,000172	1,89	0,92	IPR004873	BURP	RecName: Full=Polygalacturonase-1 non-catalytic subunit beta; AltName: Full=AroGP1; AltName: Full=Polygalacturonase converter; Short=PG converter; Flags: Precursor
CCN_CSN_000293	687	37	9	0,000172	4,11	2,04			RecName: Full=Protein TIME FOR COFFEE
CCN_CSN_000744	537	57	22	0,000174	2,59	1,37	IPR001381	Dehydroquinase class I	RecName: Full=Bifunctional 3-dehydroquinate dehydratase/shikimate dehydrogenase, chloroplastic; Short=DHQ-SDH protein; AltName: Full=DHQase-SORase; AltName: Full=Protein EMBRYO DEFECTIVE 3004; Includes: RecName: Full=Dehydroquinate dehydratase; Short=DHQ; Includes: RecName: Full=Shikimate dehydrogenase; Short=SDH; Flags: Precursor
CCN_CSN_006198	197	38	80	0,000177	0,48	-1,07	IPR000235	Ribosomal protein S7	RecName: Full=40S ribosomal protein S5
CCN_CSN_001810	399	11	40	0,000181	0,28	-1,86	IPR005935	Diphosphomevalonate decarboxylase	RecName: Full=Diphosphomevalonate decarboxylase; AltName: Full=Mevalonate (diphospho)decarboxylase; Short=MDDase; AltName: Full=Mevalonate pyrophosphate decarboxylase

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_002301	362	2	28	0,000183	0,07	-3,81	IPR000232	Heat shock factor (HSF)-type, DNA-binding	RecName: Full=Heat shock factor protein HSF30; AltName: Full=Heat shock transcription factor 30; Short=HSTF 30; AltName: Full=Heat stress transcription factor
CCN_CSN_000512	597	13	43	0,00019	0,30	-1,73	IPR000210	BTB/POZ-like	RecName: Full=Regulatory protein NPR3; AltName: Full=BTB/POZ domain-containing protein NPR3
CCN_CSN_011177	197	13	43	0,00019	0,30	-1,73	IPR012881	Protein of unknown function DUF1685	predicted protein [Populus trichocarpa]
CCN_CSN_003748	281	6	32	0,000194	0,19	-2,42	IPR001932	Protein phosphatase 2C-like	RecName: Full=Probable protein phosphatase 2C 10; Short=AtPP2C10
CCN_CSN_000917	503	64	27	0,000195	2,37	1,25	IPR000795	Protein synthesis factor, GTP-binding	RecName: Full=Eukaryotic peptide chain release factor GTP-binding subunit ERF3A; Short=Eukaryotic peptide chain release factor subunit 3a; Short=eRF3a; AltName: Full=G1 to S phase transition protein 1 homolog
CCN_CSN_001302	448	74	34	0,000197	2,18	1,12	IPR000070	Pectinesterase, catalytic	RecName: Full=Pectinesterase 3; Short=PE 3; AltName: Full=Pectin methylesterase 3; Flags: Precursor
CCN_CSN_004145	263	47	92	0,000198	0,51	-0,97	IPR000876	Ribosomal protein S4e	RecName: Full=40S ribosomal protein S4-3
CCN_CSN_000285	692	8	35	0,000199	0,23	-2,13	noIPR	unintegrated	RecName: Full=5-3 exoribonuclease 3; AltName: Full=Protein EXORIBONUCLEASE 3
CCN_CSN_012263	114	1	42	0,000201	0,02	-5,39			
CCN_CSN_004400	253	96	50	0,000202	1,92	0,94	IPR011051	Cupin, RmlC-type	RecName: Full=2-aminoethanethiol dioxygenase; AltName: Full=Cysteamine dioxygenase
CCN_CSN_010126	933	53	100	0,000204	0,53	-0,92			PREDICTED: hypothetical protein [Vitis vinifera]
CCN_CSN_009863	63	3	28	0,000204	0,11	-3,22			RecName: Full=Mannosylglycoprotein endo-beta-mannosidase; Short=AtEBM; Short=Endo-beta-mannosidase; Contains: RecName: Full=Mannosylglycoprotein endo-beta-

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
									mannosidase 31 kDa subunit; Contains: RecName: Full=Mannosylglycoprotein endo-beta-mannosidase 28 kDa subunit; Contains: RecName: Full=Mannosylglycoprotein endo-beta-mannosidase 42 kDa subunit
CCN_CSN_000152	816	56	104	0,000205	0,54	-0,89	IPR003890	MIF4G-like, type 3	RecName: Full=Eukaryotic initiation factor iso-4F subunit p82-34; Short=eIF-(iso)4F p82-34
CCN_CSN_002432	353	21	55	0,000205	0,38	-1,39	IPR010820	Protein of unknown function DUF1421	RecName: Full=Putative uncharacterized protein DDB_G0294196
CCN_CSN_005949	204	4	29	0,000208	0,14	-2,86	IPR005366	Uncharacterised protein family UPF0172	RecName: Full=UPF0172 protein At5g55940; AltName: Full=Protein EMBRYO DEFECTIVE 2731
CCN_CSN_001432	433	10	38	0,000209	0,26	-1,93	IPR010061	Methylmalonate-semialdehyde dehydrogenase	RecName: Full=Methylmalonate-semialdehyde dehydrogenase [acylating], mitochondrial; Short=MM-ALDH; Short=MMSDH; Short=Malonate-semialdehyde dehydrogenase [acylating]; AltName: Full=Aldehyde dehydrogenase family 6 member B2; Flags: Precursor
CCN_CSN_002421	354	28	65	0,000223	0,43	-1,22	IPR019410	Nicotinamide N-methyltransferase, putative	RecName: Full=Histidine protein methyltransferase 1 homolog; AltName: Full=Methyltransferase-like protein 18
CCN_CSN_002834	327	5	30	0,000231	0,17	-2,58	IPR006598	Lipopolysaccharide-modifying protein	RecName: Full=Protein O-glucosyltransferase 1; AltName: Full=CAP10-like 46 kDa protein; AltName: Full=KTEL motif-containing protein 1; Flags: Precursor
CCN_CSN_001265	453	16	47	0,000235	0,34	-1,55	IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	RecName: Full=UDP-glycosyltransferase 75B1; AltName: Full=(Uridine 5-diphosphate-glucose:indol-3-ylacetyl)-beta-D-glucosyl transferase 1; AltName: Full=IAA-Glu synthase 1; AltName: Full=Indole-3-acetate beta-glucosyltransferase 1
CCN_CSN_002738	332	50	18	0,000239	2,78	1,47	IPR003781	CoA-binding	RecName: Full=Succinyl-CoA ligase [ADP-forming] subunit alpha-1, mitochondrial; Short=SlSCoALalpha1; Flags: Precursor

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_002323	360	9	36	0,000242	0,25	-2,00	IPR001077	O-methyltransferase, family 2	RecName: Full=Caffeic acid 3-O-methyltransferase; Short=CAOMT; Short=COMT; AltName: Full=S-adenosysl-L-methionine:caffeic acid 3-O-methyltransferase
CCN_CSN_000504	600	141	85	0,000242	1,66	0,73	IPR003439	ABC transporter-like	RecName: Full=ABC transporter F family member 1; Short=ABC transporter ABCF.1; Short=AtABCF1; AltName: Full=GCN20-type ATP-binding cassette protein GCN1
CCN_CSN_010012	44	1	40	0,000243	0,03	-5,32			RecName: Full=Putative protein TPRXL
CCN_CSN_012155	123	1	39	0,000269	0,03	-5,29	IPR001763	Rhodanese-like	conserved hypothetical protein [Ricinus communis]
CCN_CSN_002763	331	6	31	0,00027	0,19	-2,37	IPR001356	Homeobox	RecName: Full=BEL1-like homeodomain protein 3; Short=BEL1-like protein 3
CCN_CSN_000107	857	42	84	0,000271	0,50	-1,00	IPR000608	Ubiquitin-conjugating enzyme, E2	RecName: Full=Probable ubiquitin-conjugating enzyme E2 24; AltName: Full=AtPHO2; AltName: Full=Ubiquitin carrier protein 24; AltName: Full=Ubiquitin-protein ligase 24
CCN_CSN_003650	285	8	34	0,000279	0,24	-2,09	IPR000504	RNA recognition motif domain	RecName: Full=Splicing factor U2af small subunit A; AltName: Full=U2 auxiliary factor 35 kDa subunit A; AltName: Full=U2 small nuclear ribonucleoprotein auxiliary factor small subunit A; Short=U2 snRNP auxiliary factor small subunit A; AltName: Full=Zinc finger CCCH domain-containing protein 8; Short=AtC3H8
CCN_CSN_000403	644	108	60	0,000283	1,80	0,85	IPR000504	RNA recognition motif domain	RecName: Full=Polyadenylate-binding protein 2; Short=PABP-2; Short=Poly(A)-binding protein 2
CCN_CSN_009735	74	70	121	0,000287	0,58	-0,79	IPR000347	Plant metallothionein, family 15	RecName: Full=Metallothionein-like protein 2; Short=MT-2
CCN_CSN_004772	240	161	68	2,90E-04	2,37	1,24	IPR002902	Gnk2-homologous domain	RecName: Full=Cysteine-rich repeat secretory protein 38; Flags: Precursor
CCN_CSN_004945	235	26	2	0,000306	13,00	3,70	IPR007502	Helicase-associated domain	RecName: Full=Probable ATP-dependent RNA helicase DHX35; AltName: Full=DEAH box protein 35

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_000913	503	61	26	0,000312	2,35	1,23			RecName: Full=Transmembrane protein DDB_G0292058; Flags: Precursor
CCN_CSN_004129	264	32	7	0,000323	4,57	2,19	IPR005062	SAC3/GANP/Nin1/mts3/eIF-3 p25	RecName: Full=26S proteasome non-ATPase regulatory subunit RPN12A; AltName: Full=26S proteasome regulatory subunit RPN12A; AltName: Full=Regulatory particle non-ATPase 12a
CCN_CSN_002580	342	55	22	0,000327	2,50	1,32	IPR000222	Protein phosphatase 2C, manganese/magnesium aspartate binding site	RecName: Full=Probable protein phosphatase 2C 78; Short=OsPP2C78
CCN_CSN_013817	44	1	37	0,00033	0,03	-5,21			
CCN_CSN_000794	528	20	52	0,00033	0,38	-1,38	IPR003663	Sugar/inositol transporter	RecName: Full=Sugar transport protein 13; AltName: Full=Hexose transporter 13; AltName: Full=Multicopy suppressor of snf4 deficiency protein 1
CCN_CSN_002362	357	53	98	0,00034	0,54	-0,89	IPR004901	Alpha-1,4-glucan-protein synthase, UDP-forming	RecName: Full=Alpha-1,4-glucan-protein synthase [UDP-forming] 2; AltName: Full=UDP-glucose:protein transglucosylase 2; Short=UPTG 2
CCN_CSN_000462	617	124	73	0,000351	1,70	0,76	IPR002293	Amino acid/polyamine transporter I	RecName: Full=High affinity cationic amino acid transporter 1; Short=CAT-1; Short=CAT1; AltName: Full=Ecotropic retroviral leukemia receptor; AltName: Full=Ecotropic retrovirus receptor; Short=ERR; AltName: Full=Solute carrier family 7 member 1; AltName: Full=System Y+ basic amino acid transporter
CCN_CSN_009971	50	1	36	0,000366	0,03	-5,17	IPR012462	Peptidase C78, ubiquitin fold modifier-specific peptidase 1/ 2	RecName: Full=Probable Ufm1-specific protease; Short=UfSP
CCN_CSN_000405	643	25	59	0,000369	0,42	-1,24	IPR021419	Mediator complex subunit Med25, von	RecName: Full=Mediator of RNA polymerase II transcription subunit 25; AltName: Full=Mediator complex subunit 25; Short=mMED25

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
								Willebrand factor type A	
CCN_CSN_000776	532	23	56	0,000378	0,41	-1,28	IPR003657	DNA-binding WRKY	RecName: Full=Probable WRKY transcription factor 31; AltName: Full=WRKY DNA-binding protein 31
CCN_CSN_007931	154	1	26	0,000383	0,04	-4,70	IPR001471	Pathogenesis-related transcriptional factor/ERF, DNA-binding	RecName: Full=Ethylene-responsive transcription factor ERF107
CCN_CSN_008532	138	1	26	0,000383	0,04	-4,70	IPR006175	Endoribonuclease L-PSP	RecName: Full=Ribonuclease UK114; AltName: Full=14.5 kDa translational inhibitor protein; Short=p14.5; AltName: Full=Heat-responsive protein 12; AltName: Full=UK114 antigen homolog
CCN_CSN_004864	237	53	21	0,000385	2,52	1,34	IPR001471	Pathogenesis-related transcriptional factor/ERF, DNA-binding	RecName: Full=Dehydration-responsive element-binding protein 1D; Short=Protein DREB1D; AltName: Full=C-repeat/dehydration-responsive element-binding factor 4; Short=C-repeat-binding factor 4; Short=CRT/DRE-binding factor 4
CCN_CSN_004496	250	19	50	0,000388	0,38	-1,40	IPR006214	Inhibitor of apoptosis-promoting Bax1-related	RecName: Full=BI1-like protein
CCN_CSN_006169	198	2	25	0,000398	0,08	-3,64	IPR021718	Pre-mRNA 3-end-processing endonuclease polyadenylation factor C-term	RecName: Full=Cleavage and polyadenylation specificity factor subunit 3-I; AltName: Full=Cleavage and polyadenylation specificity factor 73 kDa subunit I; Short=AtCPSF73-I; Short=CPSF 73 kDa subunit I
CCN_CSN_015572	71	2	25	0,000398	0,08	-3,64			
CCN_CSN_001000	489	46	88	0,000399	0,52	-0,94	IPR013863	Vacuolar import/degradation, Vid27-related	RecName: Full=Protein CYPRO4

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_002080	378	117	68	0,000 399	1,72	0,78	IPR007052	CS-like domain	RecName: Full=Protein SGT1 homolog; Short=OsSGT1; AltName: Full=Suppressor of G2 allele of SKP1 homolog
CCN_CSN_003360	299	90	145	0,000 401	0,62	-0,69	IPR008030	NmrA-like	RecName: Full=Isoflavone reductase homolog; AltName: Full=CP100
CCN_CSN_001700	409	36	10	0,000 412	3,60	1,85	IPR001461	Peptidase A1	RecName: Full=Aspartic proteinase nepenthesin-1; AltName: Full=Nepenthesin-I; Flags: Precursor
CCN_CSN_000657	558	194	130	0,000 429	1,49	0,58	IPR003657	DNA-binding WRKY	RecName: Full=Probable WRKY transcription factor 33; AltName: Full=WRKY DNA-binding protein 33
CCN_CSN_005206	226	12	39	0,000 431	0,31	-1,70	IPR002659	Glycosyl transferase, family 31	RecName: Full=Probable beta-1,3-galactosyltransferase 17
CCN_CSN_004133	264	5	28	0,000 443	0,18	-2,49	IPR007855	RNA-dependent RNA polymerase, eukaryotic-type	RecName: Full=RNA-dependent RNA polymerase 1; Short=AtRDRP1; AltName: Full=RNA-directed RNA polymerase 1
CCN_CSN_010404	355	5	28	0,000 443	0,18	-2,49			unnamed protein product [Vitis vinifera]
CCN_CSN_012339	108	14	42	0,000 445	0,33	-1,58			predicted protein [Populus trichocarpa]
CCN_CSN_007979	153	22	54	0,000 446	0,41	-1,30	IPR002222	Ribosomal protein S19/S15	RecName: Full=40S ribosomal protein S15-1
CCN_CSN_001289	450	7	31	0,000 453	0,23	-2,15	IPR003347	Transcription factor jumonji/aspartyl beta-hydroxylase	RecName: Full=Lysine-specific demethylase lid; AltName: Full=Histone demethylase lid; AltName: Full=Jumonji/ARID domain-containing protein lid; AltName: Full=Protein little imaginal disks; AltName: Full=Retinoblastoma-binding protein 2 homolog
CCN_CSN_001766	403	31	7	0,000 453	4,43	2,15	IPR000644	Cystathionine beta-synthase, core	RecName: Full=CBS domain-containing protein CBSX5
CCN_CSN_000318	670	16	45	0,000 453	0,36	-1,49	IPR000357	HEAT	RecName: Full=Importin-5; Short=Imp5; AltName: Full=Importin subunit beta-3; AltName: Full=Karyopherin beta-3; AltName: Full=Ran-binding protein 5; Short=RanBP5

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_000751	536	64	29	0,00046	2,21	1,14	IPR001554	Glycoside hydrolase, family 14	RecName: Full=Inactive beta-amylase 9; AltName: Full=1,4-alpha-D-glucan maltohydrolase; AltName: Full=Inactive beta-amylase 3
CCN_CSN_003720	282	276	365	0,000468	0,76	-0,40	IPR010399	Tify	RecName: Full=Protein TIFY 10A; AltName: Full=Jasmonate ZIM domain-containing protein 1
CCN_CSN_003731	282	1	25	0,000473	0,04	-4,64	IPR004331	SPX, N-terminal	RecName: Full=SPX domain-containing protein 2; AltName: Full=Protein SPX DOMAIN GENE 2; Short=AtSPX2
CCN_CSN_001160	467	34	9	0,000476	3,78	1,92	IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	RecName: Full=Sterol 3-beta-glucosyltransferase; AltName: Full=Autophagy-related protein 26
CCN_CSN_000262	706	168	239	0,000478	0,70	-0,51	IPR001094	Flavodoxin	RecName: Full=NADPH--cytochrome P450 reductase; Short=CPR; Short=P450R
CCN_CSN_002204	370	18	83	4,89E-04	0,22	-2,21	IPR001563	Peptidase S10, serine carboxypeptidase	RecName: Full=Serine carboxypeptidase-like 10; Flags: Precursor
CCN_CSN_002613	340	3	25	0,000492	0,12	-3,06	IPR000368	Sucrose synthase	RecName: Full=Sucrose synthase; AltName: Full=Nodulin-100; Short=N-100; AltName: Full=Sucrose-UDP glucosyltransferase
CCN_CSN_005399	221	25	3	0,000492	8,33	3,06	IPR000823	Plant peroxidase	RecName: Full=Peroxidase 51; Short=Atperox P51; AltName: Full=ATP37; Flags: Precursor
CCN_CSN_000244	715	77	14	4,99E-04	5,50	2,46	IPR000767	Disease resistance protein	RecName: Full=Disease resistance protein At4g27190
CCN_CSN_000180	776	58	25	0,000499	2,32	1,21	noIPR	unintegrated	RecName: Full=Paired amphipathic helix protein Sin3-like 4
CCN_CSN_001424	434	11	37	0,0005	0,30	-1,75	IPR000695	ATPase, P-type, H+ transporting proton pump	RecName: Full=Calcium-transporting ATPase 9, plasma membrane-type; AltName: Full=Ca(2+)-ATPase isoform 9
CCN_CSN_009915	59	1	33	0,00051	0,03	-5,04	IPR001377	Ribosomal protein S6e	RecName: Full=40S ribosomal protein S6-B
CCN_CSN_000216	740	72	35	0,000518	2,06	1,04	IPR001373	Cullin, N-terminal	RecName: Full=Cullin-1

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_004344	256	24	2	0,00052	12,00	3,58	IPR007857	Skb1 methyltransferase	RecName: Full=Probable protein arginine N-methyltransferase 6
CCN_CSN_005734	211	2	24	0,00052	0,08	-3,58	IPR001611	Leucine-rich repeat	RecName: Full=TMV resistance protein N
CCN_CSN_011554	165	2	24	0,00052	0,08	-3,58			
CCN_CSN_000710	543	29	6	0,000526	4,83	2,27	IPR000270	Phox/Bem1p	RecName: Full=Meiotically up-regulated gene 70 protein
CCN_CSN_001647	414	6	29	0,000526	0,21	-2,27	IPR000858	S-locus glycoprotein	RecName: Full=G-type lectin S-receptor-like serine/threonine-protein kinase At5g24080; Flags: Precursor
CCN_CSN_005560	216	6	29	0,000526	0,21	-2,27			RecName: Full=Zinc finger homeobox protein 4; AltName: Full=Zinc finger homeodomain protein 4; Short=ZFH-4
CCN_CSN_000427	630	15	43	0,00053	0,35	-1,52	IPR005378	Vacuolar protein sorting-associated protein 35	RecName: Full=Vacuolar protein sorting-associated protein 35; AltName: Full=Vesicle protein sorting 35
CCN_CSN_004024	269	4	26	0,000533	0,15	-2,70	IPR005175	Domain of unknown function DUF296	RecName: Full=Putative DNA-binding protein ESCAROLA
CCN_CSN_002110	376	8	32	0,00055	0,25	-2,00			RecName: Full=Uncharacterized protein DDB_G0271670; Flags: Precursor
CCN_CSN_005235	225	59	26	0,00057	2,27	1,18	IPR001623	Heat shock protein DnaJ, N-terminal	RecName: Full=Chaperone protein DnaJ
CCN_CSN_010168	605	59	26	0,00057	2,27	1,18	IPR012416	Calmodulin binding protein-like	calmodulin-binding protein [Populus euphratica]
CCN_CSN_009886	61	1	32	0,000572	0,03	-5,00	IPR003448	Molybdopterin biosynthesis MoaE	RecName: Full=Molybdopterin synthase catalytic subunit; AltName: Full=Molybdenum cofactor synthesis protein 2 large subunit; AltName: Full=Molybdenum cofactor synthesis protein 2B; Short=MOCS2B

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_008443	141	10	35	0,000579	0,29	-1,81	IPR002119	Histone H2A	RecName: Full=Probable histone H2AXb; AltName: Full=HTA3
CCN_CSN_002032	381	56	24	0,000591	2,33	1,22	IPR000504	RNA recognition motif domain	RecName: Full=RNA-binding post-transcriptional regulator csx1
CCN_CSN_015468	104	81	42	0,000607	1,93	0,95			hypothetical protein TcasGA2_TC002334 [Tribolium castaneum]
CCN_CSN_000100	875	63	29	0,000616	2,17	1,12	IPR007196	CCR4-Not complex component, Not1	RecName: Full=CCR4-NOT transcription complex subunit 1; AltName: Full=CCR4-associated factor 1; AltName: Full=Negative regulator of transcription subunit 1 homolog; Short=NOT1H; Short=hNOT1
CCN_CSN_010861	237	27	5	0,000617	5,40	2,43	IPR004274	NLI interacting factor	PREDICTED: hypothetical protein [Vitis vinifera]
CCN_CSN_012558	93	5	27	0,000617	0,19	-2,43			predicted protein [Populus trichocarpa]
CCN_CSN_000351	653	30	7	0,000636	4,29	2,10	IPR000330	SNF2-related	RecName: Full=F-box protein At3g54460
CCN_CSN_001666	412	30	7	0,000636	4,29	2,10	IPR005202	Transcription factor GRAS	RecName: Full=Scarecrow-like protein 14; Short=AtSCL14; AltName: Full=GRAS family protein 2; Short=AtGRAS-2
CCN_CSN_005902	206	7	30	0,000636	0,23	-2,10	IPR000209	Peptidase S8/S53, subtilisin/kexin/sedolisin	RecName: Full=Tripeptidyl-peptidase 2; Short=TPP-2; AltName: Full=Tripeptidyl aminopeptidase; AltName: Full=Tripeptidyl-peptidase II; Short=TPP-II
CCN_CSN_008249	147	1	31	0,000643	0,03	-4,95	IPR001932	Protein phosphatase 2C-like	RecName: Full=Probable protein phosphatase 2C 58; Short=AtPP2C58
CCN_CSN_002953	320	52	94	0,000664	0,55	-0,85	IPR002610	Peptidase S54, rhomboid	RecName: Full=Inactive rhomboid protein 1; Short=iRhom1; AltName: Full=Rhomboid family member 1
CCN_CSN_000655	559	43	82	0,000666	0,52	-0,93	IPR008862	T-complex 11	RecName: Full=T-complex protein 11-like protein 1
CCN_CSN_002632	338	3	24	0,000666	0,13	-3,00	IPR007946	A1 cistron-splicing factor, AAR2	RecName: Full=Uncharacterized protein F10B5.2

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_005228	225	24	3	0,000666	8,00	3,00	IPR001375	Peptidase S9, prolyl oligopeptidase, catalytic domain	RecName: Full=Dipeptidyl peptidase family member 6
CCN_CSN_007048	173	3	24	0,000666	0,13	-3,00			RecName: Full=Arginine and glutamate-rich protein 1
CCN_CSN_011080	208	3	24	0,000666	0,13	-3,00	IPR012442	Protein of unknown function DUF1645	PREDICTED: hypothetical protein [Vitis vinifera]
CCN_CSN_015531	80	24	3	0,000666	8,00	3,00			
CCN_CSN_001083	477	23	54	0,000699	0,43	-1,23	IPR001917	Aminotransferase, class-II, pyridoxal-phosphate binding site	RecName: Full=Serine palmitoyltransferase 2; AltName: Full=Long chain base biosynthesis protein 2; Short=LCB 2; AltName: Full=Long chain base biosynthesis protein 2a; Short=LCB2a; AltName: Full=Serine-palmitoyl-CoA transferase 2; Short=SPT 2
CCN_CSN_001057	480	11	36	0,0007	0,31	-1,71	IPR006094	FAD linked oxidase, N-terminal	RecName: Full=Reticuline oxidase-like protein; Flags: Precursor
CCN_CSN_009391	98	124	184	0,000709	0,67	-0,57	IPR004926	Late embryogenesis abundant protein, group 3	RecName: Full=Indole-3-acetic acid-induced protein ARG2
CCN_CSN_001889	393	51	21	0,000719	2,43	1,28	IPR006082	Phosphoribulokinase	RecName: Full=Phosphoribulokinase, chloroplastic; Short=PRK; Short=PRKase; AltName: Full=Phosphopentokinase; Flags: Precursor
CCN_CSN_001734	406	79	41	0,000721	1,93	0,95	IPR002735	Translation initiation factor IF2/IF5	RecName: Full=Eukaryotic translation initiation factor 5; Short=eIF-5
CCN_CSN_001590	419	13	39	0,000723	0,33	-1,58	IPR000795	Protein synthesis factor, GTP-binding	RecName: Full=Eukaryotic translation initiation factor 2 subunit 3; AltName: Full=Eukaryotic translation initiation factor 2 subunit gamma; Short=eIF-2-gamma

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_005828	208	30	1	0,000725	30,00	4,91	IPR003441	No apical meristem (NAM) protein	RecName: Full=NAC domain-containing protein 90; Short=ANAC090
CCN_CSN_009460	94	1	30	0,000725	0,03	-4,91	IPR013090	Phospholipase A2, active site	RecName: Full=Probable phospholipase A2 homolog 1; Flags: Precursor
CCN_CSN_013331	56	1	30	0,000725	0,03	-4,91			hypothetical protein ARALYDRAFT_475711 [Arabidopsis lyrata subsp. lyrata]
CCN_CSN_000177	779	48	19	0,000733	2,53	1,34	IPR000225	Armadillo	RecName: Full=U-box domain-containing protein 44; AltName: Full=Plant U-box protein 44; AltName: Full=Protein SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE 1
CCN_CSN_002073	379	15	42	0,000736	0,36	-1,49	IPR000719	Protein kinase, catalytic domain	RecName: Full=CBL-interacting serine/threonine-protein kinase 14; AltName: Full=SNF1-related kinase 3.15; AltName: Full=SOS2-like protein kinase PKS24; AltName: Full=Serine/threonine-protein kinase SR1; Short=AtSR1
CCN_CSN_000585	578	42	80	0,00079	0,53	-0,93			RecName: Full=Protein TIME FOR COFFEE
CCN_CSN_003486	292	114	68	0,000795	1,68	0,75	IPR005834	Haloacid dehalogenase-like hydrolase	RecName: Full=Phosphate metabolism protein 8
CCN_CSN_004746	241	34	10	0,000812	3,40	1,77	IPR000719	Protein kinase, catalytic domain	RecName: Full=Probable serine/threonine-protein kinase Cx32, chloroplastic; Flags: Precursor
CCN_CSN_000590	576	55	97	0,000836	0,57	-0,82	IPR002625	Smr protein/MutS2 C-terminal	RecName: Full=NEDD4-binding protein 2; Short=N4BP2; AltName: Full=BCL-3-binding protein
CCN_CSN_001732	406	12	37	0,000843	0,32	-1,62	IPR001461	Peptidase A1	RecName: Full=Aspartic proteinase Asp1; Short=OSAP1; Short=OsAsp1; AltName: Full=Nucellin-like protein; Flags: Precursor
CCN_CSN_010471	333	37	12	0,000843	3,08	1,62	IPR003729	Domain of unknown function DUF151	unnamed protein product [Vitis vinifera]
CCN_CSN_000010	1117	101	155	0,000848	0,65	-0,62	IPR001394	Peptidase C19, ubiquitin	RecName: Full=Ubiquitin carboxyl-terminal hydrolase 12; AltName: Full=Deubiquitinating

Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
								carboxyl-terminal hydrolase 2	enzyme 12; Short=AtUBP12; AltName: Full=Ubiquitin thiolesterase 12; AltName: Full=Ubiquitin-specific-processing protease 12
CCN_CSN_003299	302	26	5	0,000861	5,20	2,38	IPR006047	Glycosyl hydrolase, family 13, catalytic domain	RecName: Full=Alpha-amylase; AltName: Full=1,4-alpha-D-glucan glucanohydrolase; Flags: Precursor
CCN_CSN_010574	302	89	49	0,000861	1,82	0,86			PREDICTED: uncharacterized protein LOC100795977 [Glycine max]
CCN_CSN_008754	131	46	18	0,000864	2,56	1,35	IPR000626	Ubiquitin	RecName: Full=Polyubiquitin; Contains: RecName: Full=Ubiquitin; Flags: Precursor
CCN_CSN_009660	80	27	59	0,000868	0,46	-1,13	IPR010800	Glycine rich protein	RecName: Full=Glycine-rich protein DC7.1; Flags: Precursor
CCN_CSN_005854	207	7	29	0,000895	0,24	-2,05	IPR001806	Ras GTPase	RecName: Full=Ras-related protein Rab7
CCN_CSN_001837	397	22	2	0,000906	11,00	3,46	IPR004827	Basic-leucine zipper (bZIP) transcription factor	RecName: Full=Light-inducible protein CPRF2; AltName: Full=Common plant regulatory factor 2; Short=CPRF-2
CCN_CSN_002106	377	22	2	0,000906	11,00	3,46			RecName: Full=Leucine-rich repeat extensin-like protein 1; Short=AtLRX1; Short=LRR/EXTENSIN1; AltName: Full=Cell wall hydroxyproline-rich glycoprotein; Flags: Precursor
CCN_CSN_002238	367	22	2	0,000906	11,00	3,46	IPR001461	Peptidase A1	RecName: Full=Basic 7S globulin; AltName: Full=SBg7S; Short=Bg; Contains: RecName: Full=Basic 7S globulin high kDa subunit; Contains: RecName: Full=Basic 7S globulin low kDa subunit; Flags: Precursor
CCN_CSN_003606	287	22	2	0,000906	11,00	3,46	IPR005804	Fatty acid desaturase, type 1	RecName: Full=Omega-6 fatty acid desaturase, chloroplastic; Flags: Precursor
CCN_CSN_003911	274	22	2	0,000906	11,00	3,46	IPR007303	TIP41-like protein	RecName: Full=TIP41-like protein
CCN_CSN 010984	219	3	23	0,000908	0,13	-2,94	IPR001395	Aldo/keto reductase	PREDICTED: hypothetical protein [Vitis vinifera]

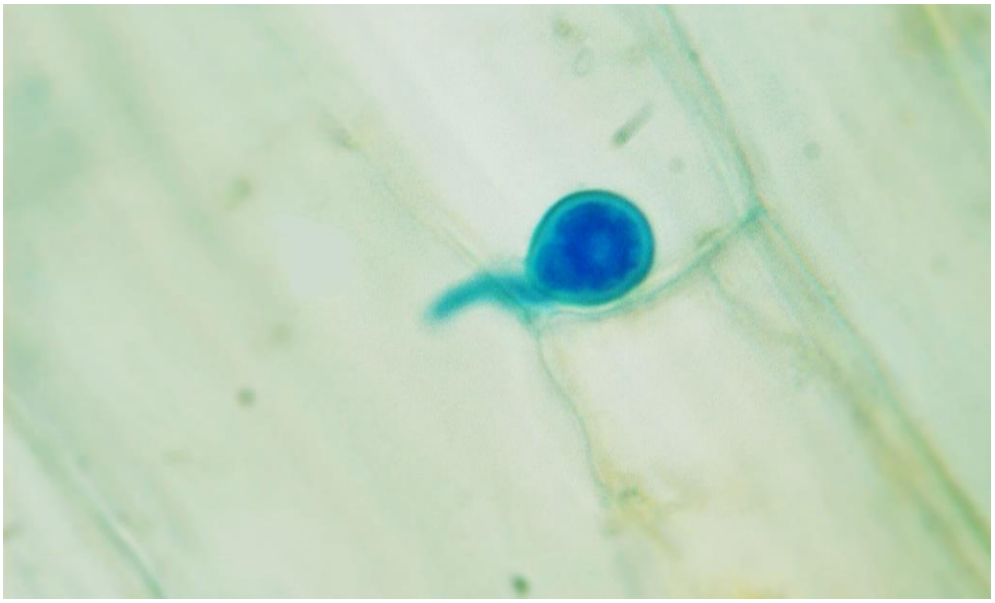
Contig name	N° a.a.	Ccn reads	Csn reads	*P* value	Ccn/Csn reads ratio	Log_2 (fold change)	Interpro ID	Interpro best hit description	BLAST best hit
CCN_CSN_002235	367	22	1	0,000923	22,00	4,46	IPR001077	O-methyltransferase, family 2	RecName: Full=Caffeic acid 3-O-methyltransferase 1; Short=CAOMT-1; Short=COMT-1; AltName: Full=S-adenosysl-L-methionine:caffeic acid 3-O-methyltransferase 1
CCN_CSN_006153	199	22	1	0,000923	22,00	4,46	IPR019315	Kinase phosphorylation domain	RecName: Full=Multiple myeloma tumor-associated protein 2 homolog
CCN_CSN_012039	132	1	22	0,000923	0,05	-4,46			
CCN_CSN_015588	67	1	28	0,000931	0,04	-4,81			
CCN_CSN_009154	112	9	32	0,000941	0,28	-1,83	IPR000231	Ribosomal protein L30e	RecName: Full=60S ribosomal protein L30
CCN_CSN_001790	401	53	23	0,000948	2,30	1,20	IPR003613	U box domain	RecName: Full=U-box domain-containing protein 28; AltName: Full=Plant U-box protein 28
CCN_CSN_002072	379	53	23	0,000948	2,30	1,20	IPR000222	Protein phosphatase 2C, manganese/magnesium aspartate binding site	RecName: Full=Probable protein phosphatase 2C 27; Short=AtPP2C27
CCN_CSN_000808	525	99	57	0,000966	1,74	0,80	IPR000504	RNA recognition motif domain	RecName: Full=Splicing factor U2af large subunit B; AltName: Full=NpU2AF65b; AltName: Full=U2 auxiliary factor 65 kDa subunit B; AltName: Full=U2 small nuclear ribonucleoprotein auxiliary factor large subunit B; Short=U2 snRNP auxiliary factor large subunit B
CCN_CSN_003070	314	35	11	0,000981	3,18	1,67	IPR000719	Protein kinase, catalytic domain	RecName: Full=Mitogen-activated protein kinase 7; Short=AtMPK7; Short=MAP kinase 7
CCN_CSN_005003	232	11	35	0,000981	0,31	-1,67	IPR003617	Transcription elongation factor, TFIIS/CRSP70, N-terminal, sub-type	RecName: Full=Transcription elongation factor B polypeptide 3; AltName: Full=Elongin-A; AltName: Full=RNA polymerase II transcription factor SIII subunit A; AltName: Full=dEloA

Supplementary material 7. Common up-regulated DEGs in *Castanea crenata* and *Castanea sativa* upon pathogen inoculation (*P* value<1E-3).Genes were associated into the functional categories in the left column.

Predicted function	Gene
Phospholipid signaling	Phospholipase D alpha 1 (precursor)
Regulation of plant immune response	Lipoxygenase A
	NAC domain-containing protein 2
	Medium-chain-fatty-acid--CoA ligase
	Ethylene-responsive transcription factor 1
Regulation of drought stress	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic
Anti-fungal enzymes	Osmotin-like protein OSM34 (precursor)
Anti-fungal metabolite synthesis	Probable cysteine desulfurase
Cell wall synthesis	Cysteine-rich repeat secretory protein 38 (precursor)
	Expansin-like B1 (precursor)
Response to drought stress	Ethylene-responsive transcription factor ABR1
	Dehydrin DHN2
	Sucrose synthase 2
Stress recovery	Formate dehydrogenase, mitochondrial (precursor)

Chapter IV

Expression analysis of genes associated with *Castanea - Phytophthora cinnamomi* interaction



The work presented in this chapter will be published in the following research publication:

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In this research paper Carmen Santos participated in the experimental design, gene selection, molecular biology, data analysis and paper writing.

Abstract

The most dangerous pathogen affecting the production of chestnuts is *Phytophthora cinnamomi* that causes root rot, also known as ink disease. Little information has been acquired in chestnut on the molecular defence strategies against this pathogen. The expression of eight candidate genes potentially involved in the defense to *P. cinnamomi* was quantified by digital PCR in *Castanea* genotypes showing different susceptibility to the pathogen. Seven of the eight candidate genes displayed differentially expressed levels depending on genotype and time-point after inoculation. *Cast_Gnk2-like* revealed to be the most expressed gene across all experiments and the one that best discriminates between susceptible and resistant genotypes. Taken together results suggest that basal defense mechanisms may be involved in the interaction of the resistant *Castanea crenata* with *P. cinnamomi*. A lower and delayed expression of the eight studied genes was found in the susceptible *Castanea sativa*, which may be related with the establishment and spread of the disease in this species. A model integrating the obtained results is presented.

Keywords: *Castanea*, *Phytophthora cinnamomi*, ink disease, plant biotic interactions, digital PCR

Introduction

The European chestnut tree (*Castanea sativa* Mill.), also known as sweet chestnut, is a species of flowering tree of the Fagaceae family, native to Europe and Asia Minor and widely cultivated throughout the temperate world. In the Mediterranean region, the European chestnut has a significant economic role mainly because of the high quality of its nuts, which production was about 117 207 tones in 2013 (FAOSTAT, 2016, faostat.fao.org).

The ubiquitous oomycete *Phytophthora cinnamomi* is the most severe pathogen affecting European chestnut, causing root rot and death, resulting

in large losses in chestnut production. In Portugal, there was a decrease of 27.3% in the distribution area of chestnut between 2002-2004, due to *P. cinnamomi* infections (Martins et al. 2007). *P. cinnamomi* has an exceptionally wide host range, being able to destroy thousands of plant species worldwide and causing devastating impacts in natural ecosystems, agriculture, horticulture, forestry and in the nursery industry (Cahill et al. 2008; Hardham, 2005; Kamoun et al. 2014; Robin et al. 2012). Among chestnuts, the Japanese chestnut (*Castanea crenata* Sieb. et Zucc) and the Chinese chestnut (*Castanea mollissima* Bl.) show resistance to *P. cinnamomi* (Crandall et al. 1945). Therefore, these East Asian species have been used in chestnut breeding programs as donors of resistance to root rot in Europe since the last century.

Ten years ago, a breeding program was initiated in Portugal to introgress resistance genes of Asian species (C. *mollissima* and *C. crenata*) into *C. sativa*, by controlled crosses (Costa et al. 2011). Nevertheless, the knowledge about the molecular mechanism driving chestnut resistance to the ink disease is still scarce. To overcome such limitation, a study has been conducted to identify candidate genes differentially expressed in roots of the susceptible species, *C. sativa*, and the resistant one *C. crenata* observed after *P. cinnamomi* inoculation (Serrazina et al 2015 and Chapter III). While this approach constituted a valuable contribution to the *Castanea* genomic resources, more studies are needed to validate the candidate genes previously identified.

The aim of this study is to evaluate the expression of genes potentially involved in the resistance to *P. cinnamomi* in *C. sativa* and *C. crenata*, as well as in four hybrids (three *C. sativa* x *C. crenata* genotypes and a *C. sativa* x *C. mollissima*) with different responses to *P. cinnamomi*, produced by the Portuguese chestnut breeding program.

Among the different methods available to quantify gene expression in plants, digital PCR (dPCR) is emerging as an absolute quantification method with

high precision, sensitivity and specificity (Majumdar et al. 2015). This new technology has been mainly used for biomedicine research (Kinz et al. 2015; Salvi et al. 2015; Sefrioui et al. 2015; Stabley et al. 2015). However, some studies in plant science using dPCR have also been recently released (Bahder et al. 2016; Ge et al. 2016; Kadam et al. 2016; Stevanato and Biscarini, 2016).

Material and Methods

Plant material and *P. cinnamomi* inoculation

Six chestnut genotypes showing different levels of resistance after inoculation with the pathogen were used in this work. In table 1 a characterization of the resistance levels of each genotype is provided. *Castanea crenata* (resistant) and *Castanea sativa* (susceptible) genotypes were provided by TRAGSA nursery (Grupo TRAGSA-SEPI, Maceda, Spain) and correspond to the genotypes used by Serrazina et al (2015) for root transcriptomes sequencing in Chapter III. Four hybrid genotypes with different responses to *P. cinnamomi* were selected from the on-going chestnut breeding program (Santos et al. 2015 and Chapter II): three *C. sativa* x *C. crenata* hybrids (SC55, SC914 and SC903) and a *C. sativa* x C. *mollissima* hybrid (SM904), selected as a resistance control.

All plant material used in this study was multiplied by *in vitro* propagation (Supplementary Figure 1). First, individual shoots from mother trees were established and multiplied on Murashige and Skoog medium (half concentration of NH_4NO_3 and KNO_3), supplemented with 1g/L and 0.1g/L benzylaminopurine, respectively, 30g/L sucrose and 8g/L phyto-agar. Elongated shoots were transferred to Murashige and Skoog medium described above (without phyto-hormones) plus 3g/L charcoal for 7-10 days. Rooting phase consists on dipping elongated shoots into 1g/L indolebutyric acid for 1 min and then placed at a wet porous substrate, perlite:vermiculite (1:1), for 3 weeks. Rooted plants are transferred to pots with

peat:vermiculite:perlite (1:1:1). All propagation steps were performed under controlled conditions with temperatures ranging between 18 and 24ºC, photoperiod 16h light/8h dark.

Table 1. Characterization of the chestnut samples used in this study. Hybrid phenotyping data was assessed by Santos et al. (2015), reported on Chapter II.

Sample Name	Species	Origin	Survival's percentage	Days of Survival (average)	Level of resistance
C. sativa	*Castanea sativa*	TRAGSA	0	7	Susceptible
C. crenata	*Castanea crenata*	TRAGSA	83	92	Resistant
SM904	*C. sativa* x *C. mollissima* (F1)	Portugal	46	76	Resistant
SC55	*C. sativa* x *C. crenata* (F1)	Portugal	38	70	Resistant
SC914	*C. sativa* x *C. crenata* (F1)	Portugal	0	29	Intermediate
SC903	*C. sativa* x *C. crenata* (F1)	Portugal	0	10	Susceptible

P. cinnamomi root inoculation was performed 80 days after plant acclimatization under controlled conditions and according to Santos et al (2015), Chapter II. Briefly, *P. cinnamomi* inoculum was prepared by growing mycelia on sterilized vermiculite, which were thoroughly moistened with a solution of 200 mL V8 vegetable juice, 3g of calcium carbonate and 800 mL distilled water. Afterwards, this mixture was incubated for 3 weeks in darkness at 24°C. Inoculum was placed into the substrate of each pot at a concentration of 5% (v/v), minimizing root disturbance, and flooded for 1 hour to stimulate zoospore release, promoting the root infection and disease development. Aiming cover diverse facets of host defense response, three root biological replicates were harvested per genotype at 0 (uninfected), 24 and 48 hours post inoculation (hpi), corresponding to different stages of

pathogen colonization (Redondo et al. 2015). Roots were gently washed and separated from the aerial part, frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

Selection of Candidate Genes

Genes were selected from the 283 *C. crenata* differentially expressed genes (DEGs), previously identified by Serrazina et al. 2015 and Chapter III. Transcriptomic data sets are publicly available on the Fagaceae Genomics website (http://fagaceae.org/) and in the Short Read Archive at NCBI (http://www.ncbi.nlm.nih.gov/) with the reference PRJNA215368. In this study, gene selection parameters were: 1) DEGs with the $\log_2$ of the ratio between *C. crenata* inoculated (Cci) and non-inoculated (Ccn) reads higher than 1.5 (Log_2Cci/Ccn >1.5); 2) The correspondent DEGs in *C. sativa* transcriptomes with Log_2Csi/Csn <1.5 or absent; 3) DEGs not involved in general biological processes, such as oxidative, metabolic and transporter activities; 4) DEGs involved in defense response and categorized in pathogen recognition which usually triggers resistance signaling pathways, anti-pathogen proteins, cell wall modification proteins and transcription factors involved in the regulation of other defense related processes.

Primer and probe design

Primers and TaqMan®-Probes sequences were designed using Primer 3 software version 0.4.0 (available at http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and were synthesized by Life Technologies. Conserved domain sequences were avoided to primer design in order to increase the specificity. Primer selection parameters were set: primer size of 18-20 bp, a product size range of 100-150 bp; a primer melting temperature of 58-60ºC; primer GC content of 30-60%, primer with no more than two G/C in the last five 3' end nucleotides and no more than three G's runs within the sequences. TaqMan®-Probes design followed the same criteria, except size

between 18-30bp and melting temperatures ranging 68-70ºC. Probes were labelled with FAM or VIC dye on the 5' end and NFQ (Non-fluorescent Quencher) on the 3' end.

RNA isolation and cDNA synthesis

Total RNA from root tissue was isolated as described in Le Provost et al. 2007, without DNase treatment. mRNA was purified using the Dynabeads® mRNA Purification Kit (Life Technologies) using half volume of dynabeads and buffers, and according to the manufacturer's instructions. RNA and mRNA quality was assessed by measuring the ratios of absorbance at 260/280 and 230/280 using a nanodrop; the results obtained were, in average, $absorbance_{260/280}$=1.92 and $absorbance_{230/280}$=1.77. mRNA was used for cDNA synthesis using RevertAid H Minus Reverse Transcriptase kit (ThermoFisher Scientific). 0.5 µg of $oligo(dT)_{18}$ primer and DEPC-treated water to make 12,5 µl were added to 50 ng of mRNA and incubated at 65º C for 5 min. Then, 1x reaction buffer [250 mM Tris-HCl (pH 8.3 at 25º C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT], 20 units of ribolock RNase inhibitor, dNTP Mix (1 mM final concentration) and 200 units of RevertAid H Minus Reverse Transcriptase were added to the previous mixture and incubated 60 min at 42°C. Reverse transcriptase was inactivated by heating at 70°C for 10 min.

QuantStudio™ 3D Digital PCR

QS3D digital PCR System (Life Technologies) was used to quantify gene expression of eight *P. cinnamomi* resistance candidate genes in the roots of the six chestnut genotypes under study. 0.125 to 2.5 ng of cDNA and two TaqMan® probes (specific primers/probe mix) one labelled with FAM and the other with VIC, were added to the QS3D master mix. Each QS3D chip was loaded with 14,5 µL reaction and sealed, using an automatic chip loader (Life Technologies) and according to the manufacturer´s instructions. The QS3D

chip amplification was performed on the dual flat-block GeneAmp® PCR System 9700 thermal cycler with the following conditions: 96ºC 10 min, 60ºC 2 min and 98ºC 30 sec for 40 cycles, then 60ºC for 2 min and hold at 25ºC (avoiding chip condensation). After amplification, the chips were imaged on the QS3D Instrument, which assesses raw data and calculates the estimated concentration of the nucleic acid sequence targeted by FAM and VIC labelled probes assuming a Poisson distribution (Fazekas de St Groth, 1982). Data analysis and management were performed using QuantStudio™ 3D Analysis Suite™ software (https://apps.lifetechnologies.com/quantstudio3d/). Chip quality control was calculated based on the number of partitions that exceed the selected quality threshold (fixed automatically at 0.5) on the total number of wells filled correctly. The software automatically removed data points that did not meet the default quality threshold. Cn/µL were calculated by software taking into account the dilution factor.

To estimate the absolute copies of template molecules, present in the sample volume, the software applies a quantification algorithm based on the Poisson model. The estimated Cn/µL mean values are presented in a confidence interval at 95%. Standard deviation was calculated assuming the Poisson distribution of the data. Shapiro-Wilk test was used to confirm the type of data distribution. Comparison of gene expression between *C. sativa* and each of the other genotypes was done using the Wilcoxon-Mann-Whitney (non-parametric) test.

Results

P. cinnamomi phenotyping

The hybrid genotypes used in this study were previously phenotyped to *P. cinnamomi* susceptibility after root inoculation by Santos et al. (2015) and are reported in Chapter II. Moreover, response to *P. cinnamomi* was also evaluated for *C. sativa* and *C. crenata* genotypes showing contrasting

responses: *C. sativa* plants died one week after inoculation, while 83% of *C. crenata* plants survived to inoculation (Table 1).

Resistance candidate genes to *P. cinnamomi*

Using the gene selection parameters defined, eight candidate genes were identified (Table 2). These genes codify proteins potentially involved in diverse levels of response to *P. cinnamomi* infection: 2 pathogen recognition proteins (*Cast_LRR-RLK* and *Cast_C2CD*) which trigger resistance signaling pathways; three transcription factors (*Cast_WRKY 31*, *Cast_ABR1* and *Cast_Myb4*) involved in the regulation of other defense processes; a ubiquitination regulator (*Cast_RNF5*); a cell wall modification enzyme (*Cast_PE-2*) and an antifungal protein (*Cast_Gnk2-like*).

Table 2. Candidate genes identification. Log_2 ratio between *C. crenata* inoculated and *C. crenata* non-inoculated, *P-value* and BLAST best hit information. More details are presented in Supplemetary material 3 in Chapter III.

Gene acronyms	Log_2 (Cci/Ccn)	*P-value*	BLAST best hit (Species)
Cast_Gnk2-like	2,88	$1,13e^{-12}$	Gnk2-homologous domain, Cysteine-rich repeat secretory protein 38 (*Oryza sativa*)
Cast_PE-2	2,98	$4,90e^{-08}$	Pectinesterase 2 (*Populus trichocarpa*)
Cast_ABR1	4,64	$2,30e^{-13}$	Pathogenesis-related transcriptional factor, Ethylene-responsive transcription factor (AP2/ERF) ABR1 (*Ricinus communis*)
Cast_C2CD	2,48	$6,60e^{-05}$	C2 calcium-dependent membrane targeting, C2 domain-containing protein (*Arabidopsis thaliana*)
Cast_LRR-RLK	2,32	$6,35e^{-07}$	LRR receptor-like serine/threonine-protein kinase (*Ricinus communis*)
Cast_Myb4	2,95	$1,28e^{-08}$	SANT domain, DNA binding, Myb-related protein Myb4 (*Vitis vinefera*)
Cast_WRKY 31	1,71	$8,18e^{-06}$	WRKY transcription factor 31 (*Arabidopsis thaliana*)
Cast_RNF5	2,97	$1,19e^{-05}$	Zinc finger, RING finger protein 5 (*Lactobacillus crispatus*)

All genes selected were up-regulated after inoculation in *C. crenata* root transcriptomes (Serrazina et al. 2015, Chapter III). The *P. cinnamomi* resistance candidate genes, their respective contig name (Serrazina et al. 2015), primers and TaqMan®-Probes sequences are listed in Supplementary Table 1.

Accuracy and precision of QS3D quantification method

QuantStudio™ 3D AnalysisSuite™ software evaluates if the data on a chip is reliable based upon loading, signal, and noise features. Quality indicators (red, yellow or green flags, corresponding from low to high quality, respectively) are displayed for each chip. As an example, chips' output used to quantify *Cast_WRKY 31* and *Cast_Myb4* expression in three biological replicates (1 chip per replicate) of *C. crenata* genotype, 48hpi, are shown in (Figure 1). The continuous green color displayed in each chip confirms high quality loading (Figure 1A). Nevertheless, some condensation occurred on the corners, presented by yellow or red data points. White dots were automatically filtered out because they did not meet the default quality threshold. A random distribution of each target gene amplified (FAM, VIC or both dyes) and negative reactions (non-amplified wells) are shown (Figure 1B, C). Clustering of the scatter plots of the biological triplicates allows verifying the technical homogeneity of the results. The dilution factor was considered by the software to calculate the copy number per microliter (Cn/µL).

Chestnut gene expression profiling

Transcripts copy number variation among the three time-points for six chestnut genotypes is presented in Figure 2 and Figure 3. Cn/µL ranged from approximately 100 to 27.000, being the lowest values obtained for the basal expression of the *C. sativa* genotype and the most susceptible *C. sativa* x *C. crenata* hybrid (SC903) (Figure 2 and 3). Aside from *Cast_ABR1*,

transcription factors presented the lowest Cn/µL, particularly in the most susceptible genotypes and in the two first time points (non-inoculated and 24hpi).

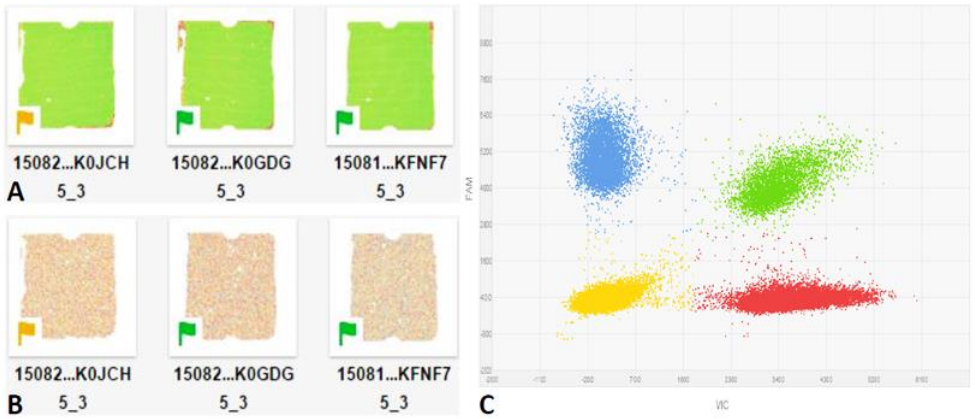

Figure 1. Representative QS3D chip views and respective plot of *Cast_WRKY 31* and *Cast_Myb4* expression for three biological replicates (*C. crenata*, 48hpi). A) Chip views depicting color by quality. B) Chip views depicting color by calls. C) Scatter plot view from merging the three biological replicates. The data points on chip (B) and plot views (C) are color-coded according to the following fluorophores' color: FAM (blue), VIC (red), FAM + VIC (green) and not amplified (yellow). Relative intensities of FAM were plotted against VIC.

The expression profiles varied depending on genotype susceptibility, mainly for the *Cast_Gnk2-like*, *Cast_PE-2*, *Cast_LRR-RLK* and *Cast_Myb4* genes (Figure 2 and 3 A, B, E and F). *Cast_Gnk2-like* revealed to be the most expressed gene, whose expression increases from the most susceptible to the most resistance genotype (Figure 2A and 3A). On the other hand, *Cast_RNF5* and *Cast_C2CD* showed to have the less variation between samples and time points. *C. crenata* presented more transcript abundance than *C. sativa* in most cases. Except for *Cast_C2CD*, *Cast_RNF5* and *Cast_ABR1*, an accumulation of transcripts from the most susceptible to the less susceptible hybrids of *C. sativa* x *C. crenata* was observed.

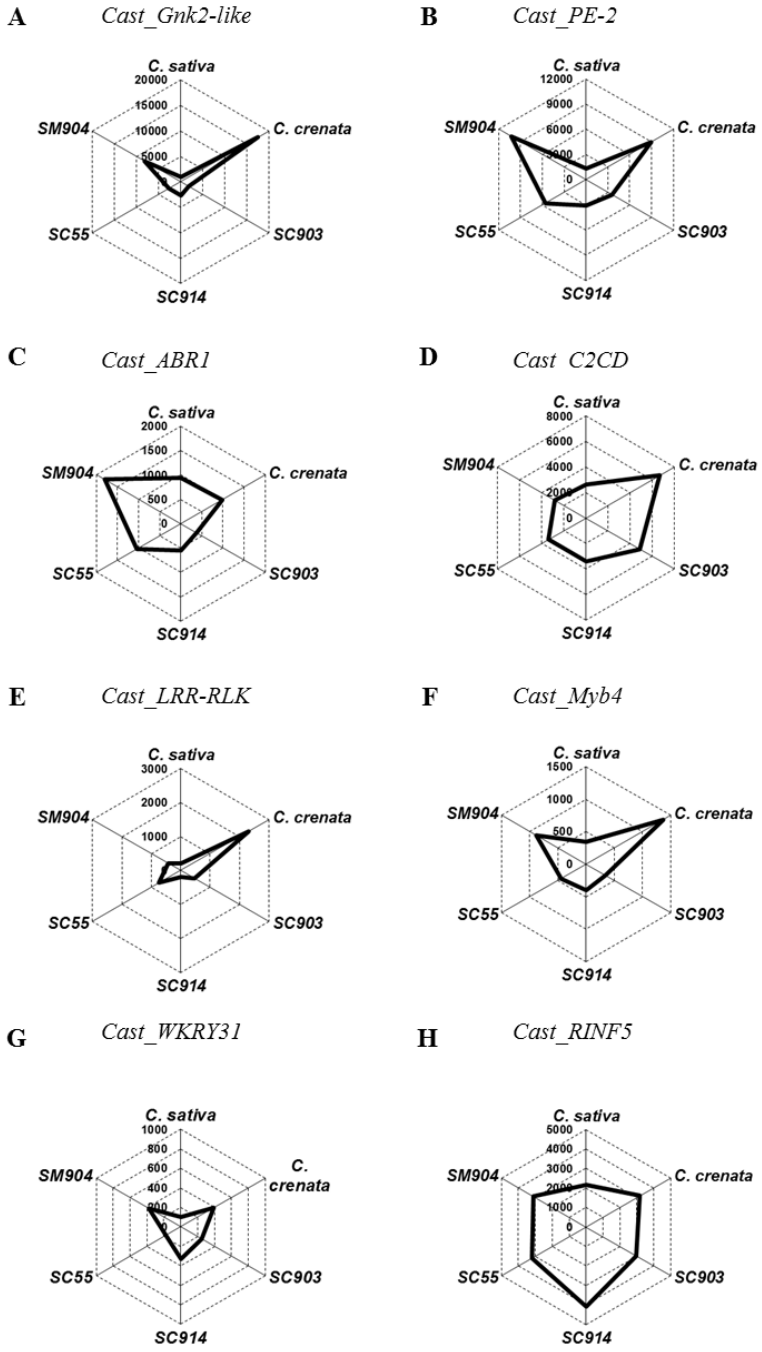

Figure 2. Radar plots of copy number/µL of the eight genes in non-inoculated roots. Starting on top and following clockwise, *C. sativa*, *C. crenata,* SC903, SC914, SC55 and SM904 genotypes are presented.

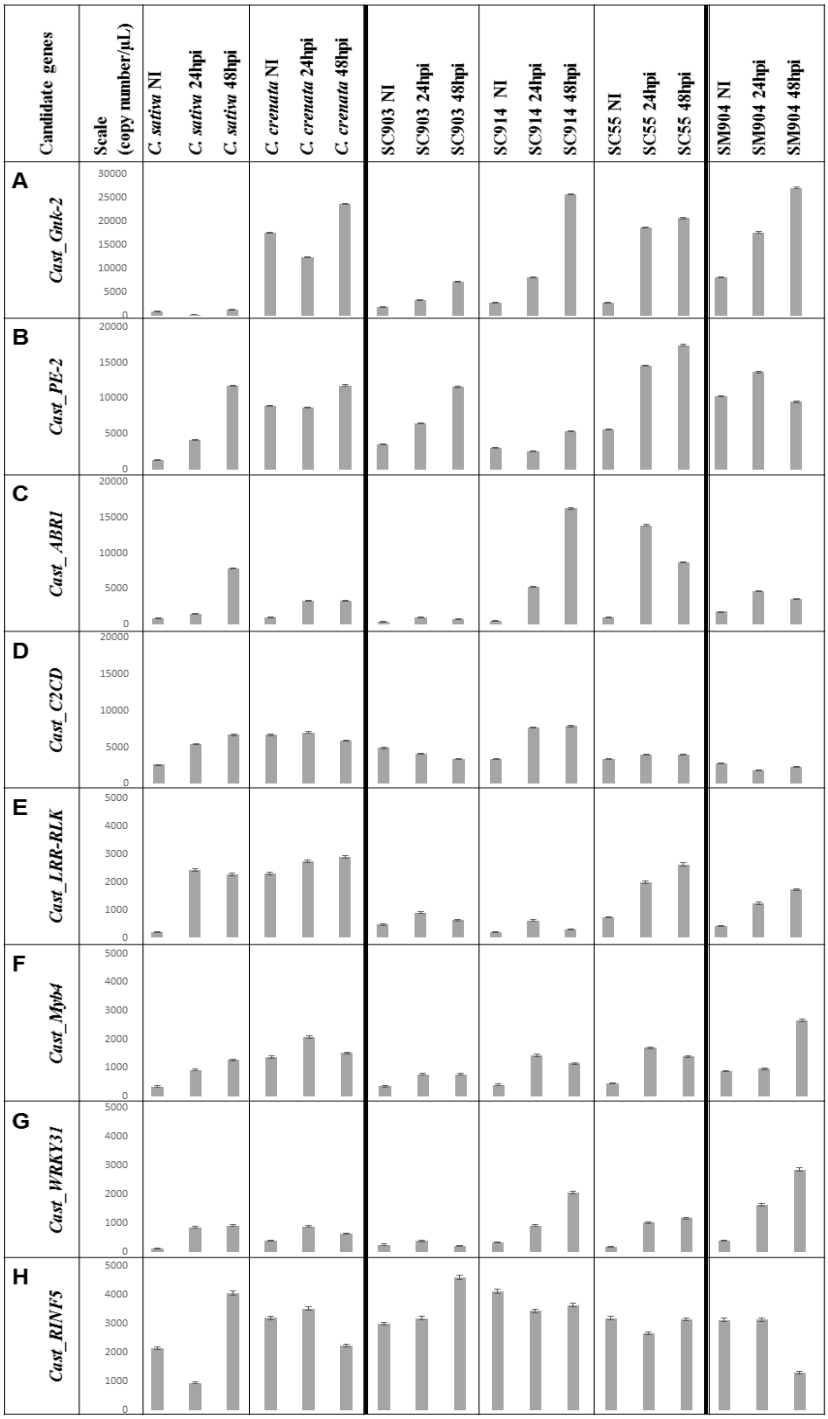

Figure 3. Variation of copy number/µL of eight genes under study. Chestnut genotypes are presented for *C. sativa* and *C. crenata*, *C. sativa x C. crenata* hybrid

genotypes from the most susceptible (SC903) to the most resistant (SC55) and the resistant *C. sativa x C. mollissima* genotype (SM904). For each genotype, Cn/µL for 0hpi (not inoculated), 24hpi and 48hpi is shown. The mean value of each bar corresponds to the quantification of biological triplicates, calculated by the software assuming a Poisson distribution; error bars correspond to standard deviations. Y axis: copies/µL; X axis: sample name x treatment, NI: non-inoculated, hpi: hours post-inoculation. A: scale adjusted to 30 000 copies/µL; B-D: scale adjusted to 20 000 copies/µL; E-F: scale adjusted to 5 000 copies/µL.

In all the analysed profiles, the expression levels of the eight candidate genes changed along the time points. Before the inoculation with the pathogen, all candidate gene transcripts accumulated more in *C. crenata* than in *C. sativa*, mainly for *Cast_Gnk2-like*, *Cast_PE-2*, *Cast_C2CD*, *Cast_LRR-RLK* and *Cast_Myb4* genes (Figure 2). These differences were significant (α=0.05) between *C. sativa* and *C. crenata*, as well as, between *C. sativa* and hybrids, for all genes under study. Nevertheless, the intermediate *C. sativa* x *C. crenata* hybrid (SC914) showed similar Cn/µL with *C. sativa* in non-inoculated samples *for Cast_PE-2*, *Cast_C2CD* and *Cast_LRR-RLK*. Except for *Cast_Gnk2-like*, *Cast_C2CD* and *Cast_LRR-RLK*, resistant *C. sativa* x C. *mollissima* hybrid (SM904) showed similar basal expression profiles to *C. crenata*.

Considering the whole experiment, there is a tendency for the accumulation of the transcripts at 48hpi. However, in the majority of cases, *Cast_Myb4* is more expressed at 24hpi than 48hpi. This difference observed between time points decreases gradually from the resistant *C. crenata* to the susceptible genotypes, reaching the point where *C. sativa* expression is higher at 48hpi (Figure 3F).

Discussion

Basic knowledge on the molecular defense mechanisms against *P. cinnamomi* infection is required in both resistant and susceptible genotypes. The expression of eight resistance candidate genes was evaluated before and after inoculation of *C. sativa*, *C. crenata* and four interspecific hybrids of the ongoing Portuguese chestnut breeding program. *C. crenata* showed the highest expression of these genes, especially in non-inoculation conditions, opposing to *C. sativa*, in which the lower transcripts abundance was measured. The results seem to show that basal defense mechanisms may explain the difference in *P. cinnamomi* resistance between *C. sativa* and *C. crenata*. Basal resistance may have evolved during host-pathogen coevolution, since *P. cinnamomi* is native to Asia (Ko et al. 1978; Zentmyer, 1988; Zhang et al. 1994) and *C. crenata* (Japanese chestnut) seem to be the ancestral of the other species of *Castanea* genus (Lang et al. 2007).

Physical and chemical barriers to *P. cinnamomi* infection

The secretion of toxic compounds is an effective defense mechanism against pathogens in plants (Montesinos, 2007; Wittstock and Gershenzon, 2002). Ginkbilobin-2 (Gnk2) is a protein secreted by *Ginkgo biloba* seeds that exhibits an antifungal activity (Sawano et al. 2007; Wang and Ng, 2000). Gnk2 has a plant-specific cysteine-rich motif DUF26 (domain of unknown function 26, also known as stress-antifungal domain: PF01657) which belongs to cysteine-rich receptor-like kinases (CRKs) (Miyakawa et al. 2014) not showing any similarity with other known antimicrobial proteins (Sawano et al. 2007, Miyakawa et al 2014). It was recently shown that Gnk2 can also activate actin-dependent cell death (Gao et al. 2015). Therefore, *Cast_Gnk2-like* may prevent pathogen growth either by its chemical properties or by inducing Hypersensitive response (HR)-related cell death.

The highest basal *Cast_Gnk2-like* expression registered suggests that *C. crenata* root surroundings may be a hostile environment for fungal and

fungal-like pathogens, such as *P. cinnamomi*. On the other hand, *C. sativa* showed a very low *Cast_Gnk2-like* expression level, even after pathogen inoculation. Considering the whole experiment, *Cast_Gnk2-like* was the most expressed gene and that best discriminates between susceptible and resistant genotypes (Figure 2A and 3A). The isolation and purification of Cast_Gnk2-like protein may have biotechnological applications, such as the development of an antimicrobial phytopharmaceutical against *P. cinnamomi*. A crucial basal defense is the formation of wall appositions that comprise a physical barrier to pathogen growth (Hardham and Blackman, 2010). The reinforcement of plant cell walls by calcium-pectate gel apposition with the involvement of pectinesterases have been shown to confer resistance to *Phytophthora* species (Kieffer, 2000; Wiethölter et al. 2003). In this study, expression levels of *Cast_PE-2* show that this enzyme may have a role on *P. cinnamomi* resistance in chestnut. Compared with *C. sativa*, *C. crenata* exhibited higher *Cast_PE-2* expression levels in all time points, mainly in the non-inoculated samples (about 10x more), suggesting that their cell walls may be more resistant to pathogen penetration. After the first pathogen contact, *Cast_PE-2* expression increases, suggesting a possible continuing apposition of pectates in cell walls, probably to inhibit further colonization. This seems to be more important in a late stage of infection (48hpi) except for the *C. sativa* x C. *mollissima* hybrid. Possibly, other resistance mechanisms may be activated earlier in this hybrid and control the infection.

Pathogen recognition and successive host response regulation

Generally, during pathogen infection, Pathogen-Associated Molecular Patterns (PAMPs) are recognized by pattern-recognition receptors (PRRs) at the plant's cell surface. The best-studied class of plant PRRs are receptor-like kinases (RLKs), which have an ectodomain of leucine-rich repeats (LRRs) involved in PAMP perception (Boller and Felix 2009; Jones and Dangl 2006; Hove et al. 2011). Resistance related LRR proteins have been

found to be involved in *Phytophthora* spp. infection response (Ballvora et al. 2002; Vossen et al. 2003; Gao et al. 2005; Mahomed and Berg 2011; Boava et al. 2011; Coelho et al. 2011). Contrasting to *C. sativa*, *C. crenata* has a much higher (about 10x more) basal *Cast_LRR-RLK* expression (Figure 2E and 3E), which may mediate a fast and effective response against *P. cinnamomi*, suggesting that this earlier recognition is part of the resistance phenotype. Furthermore, *Cast_LRR-RLK* expression increased after *P. cinnamomi* inoculation for all *Castanea* genotypes under study. Considering the previous studies on LRR biological functions in Fagaceae, *Cast_LRR-RLK* will recognize and interact with PAMPs molecules, secreted by *P. cinnamomi*, activating downstream signaling responses (Coelho et al. 2011). RLKs have an intracellular kinase domain involved in a downstream signaling via MAPK cascades which trigger defense-related pathways by transcription factors activation (Pitzschke et al. 2009; Tena et al. 2011), such as *WRKY*, *MYB* and *Ethylene-responsive transcription factors* (Dubos et al. 2010; Kim and Zhang, 2004; Oñate-Sánchez and Singh, 2002). WRKY proteins regulate pathogen- and salicylic acid (SA)-responsive genes having a pivotal role in host response to stress (Dong et al. 2003; Eulgem, 2000; Eulgem and Somssich, 2007; Shimono et al. 2012; Yang et al. 2009). In particular, the overexpression of *WRKY 31* in rice seedlings after treatment with a hemibiotrophic fungus (*Magnaporthe grisea*) was associated with blockade of pathogen invasion (Zhang et al. 2008). *Cast_WRKY 31* seems to have a role in the response of chestnut to *P. cinnamomi* infection, since its expression increased in inoculated samples when compared with non-inoculated ones, probably regulating SA-responsive genes expression. This increase seems more consistent in the more resistant hybrids.

SA induces defense responses against biotrophic pathogens (Loake and Grant, 2007; Vlot et al. 2009). High concentrations of endogenous (SA) may induce HR (Mur et al. 2008).

The balance between SA and other phytohormones is increasingly recognized as central to the outcome of plant–pathogen interactions (Zabala et al. 2009). Abcisic acid (ABA) disrupts SA-mediated response and suppresses the expression of many defense-related genes. *Ethylene-responsive transcription factor ABR1* is a negative regulator of ABA signaling pathway in *Arabidopsis thaliana* (Pandey et al. 2005) and its expression allows SA and lignin accumulation (Mohr and Cahill 2007; Zabala et al. 2009; Boatwright and Pajerowska-Mukhtar 2013). *Cast_ABR1* expression was triggered after *P. cinnamomi* inoculation, earlier in the more resistant genotypes, suggesting that ABA may be repressed after pathogen perception. In the resistant *C. crenata* genotype the relatively low increase of *Cast_ABR1* expression may due to the efficiency of other resistant mechanisms that avoid pathogen colonization, or by independence of ABA suppression for SA signaling activation.

Genes of the *MYB transcription factor* family are involved in the control of specific processes including responses to biotic stresses (Dubos et al. 2010). MYB4 has been shown to repress transcription of cinnamate 4-hydroxylase (C4H) enzyme (Hemm et al. 2001). C4H catalyze the second step of the main phenylpropanoid pathway, leading to the synthesis of lignin, pigments, and defense molecules. Inactivation of C4H allows the accumulation of SA in elicited cells (Schoch et al. 2002). The expression balance of *Cast_Myb4* in *Castanea* genotypes may regulate SA accumulation versus synthesis of phenylpropanoids. The ratio of *Cast_Myb4* expression between 24/48hpi decreased progressively from the resistant *C. crenata*, to *C. sativa* x *C. crenata* hybrids (the most resistant to the most susceptible) to the susceptible *C. sativa*. This indicates that SA signaling may be faster (24hpi) in resistant genotypes than in susceptible ones. As mentioned before, elevated concentrations of endogenous SA will induce expression of *Cast_Gnk2-like* and *Cast_WRKY31*. For resistant genotypes (*C. crenata* and SC55), after a probable early induction of SA pathways, expression of *Cast_Myb4*

decreases at 48hpi, which may allow the synthesis of lignin and other defense molecules.

In addition to MAPK cascades regulation to activate transcription factors, the defense regulation could be also calcium-dependent, since intracellular calcium increases upon pathogen recognition (Ma and Berkowitz, 2007). Calcium rapid and transient bursts act as a key second messenger in cell signaling, inducing HR to prevent pathogen colonization (Lecourieux et al. 2002, 2006; Ma and Berkowitz, 2007). C2 domains are ubiquitous structural modules that act in Ca^{2+}-dependent membrane binding. Several small C2 proteins in plants have been shown to be involved pathogen responses (Kim et al. 2003; Lecourieux et al. 2006; Wang et al. 2009). The expression profile of *Cast_C2 domain* is not in accordance with the resistant phenotypes. Nevertheless, the basal expression of *Cast_C2 domain* in *C. crenata* is noteworthy (Figure 2D and 3D). The role of *Cast_C2 domain* to *P. cinnamomi* infection warrants further investigation. Likewise, *Cast_RNF5* showed to have the least variation between samples and time points (Figure 3H), suggesting that *Cast_RNF5* is not a key regulator in the chestnut response to *P. cinnamomi* infection, at least in the first 48hpi.

Hypothetical *P. cinnamomi* response mechanism in *Castanea*

The expression profiles obtained suggest that susceptible and resistant plants may share the same response mechanisms. Despite, resistant plants show a much higher constitutive expression of the tested candidate genes without inoculation. A model for *Castanea* spp. response to *P. cinnamomi* infection is proposed (Figure 4): resistant genotypes present a higher basal expression of genes that may be part of a basal defense mechanism that prepare and protect the plant in advance to *P. cinnamomi* infection by secreting antifungal proteins and having stronger cell walls even before the contact with the pathogen. If *P. cinnamomi* overcomes those chemical and physical barriers, specific pathogen recognition proteins are earlier and more

expressed in the resistant genotypes when compared to the susceptible ones. Thereafter, the transcription of the host will probably be reprogrammed via signal transduction and SA signaling. HR-related cell death is probably activated and cell walls may be reinforced in non-infected tissues, preventing further colonization.

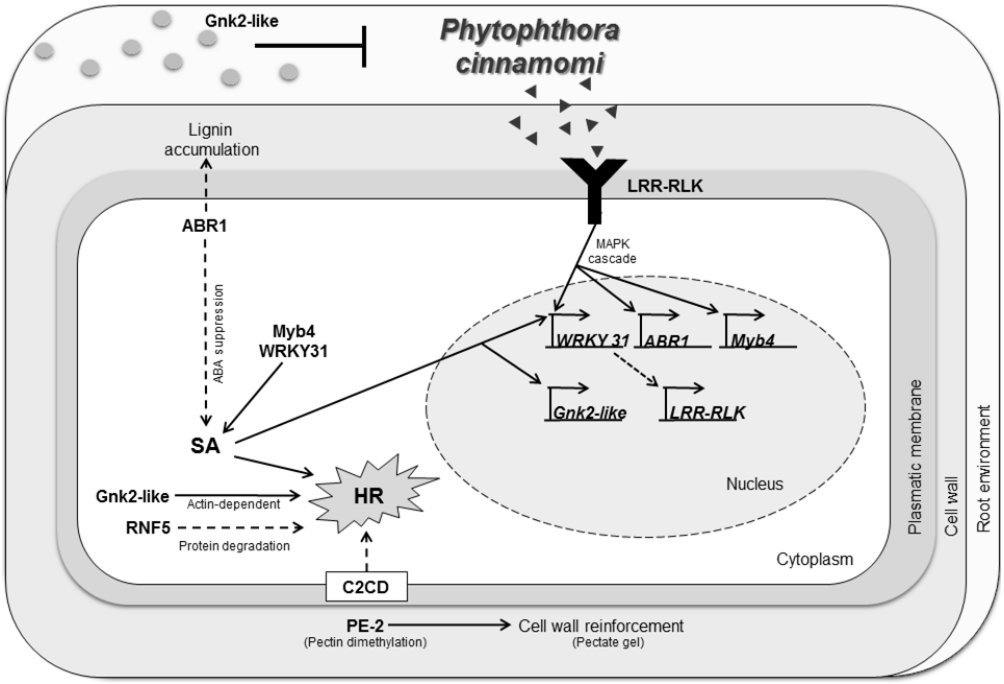

Figure 4. Hypothetical molecular mechanism for *P. cinnamomi* response. Physiochemical barriers, antifungal proteins secretion (*Cast_Gnk2-like*) and stronger cell walls (by action of *Cast_PE-2, Cast_ABR1*) respectively, may inhibit *P. cinnamomi* growth and infection. If *P. cinnamomi* overcome those barriers, specific pathogen recognition may occur, by *Cast_LRR-RLK*. Hence, host transcription is reprogramed via MAPK cascades and SA signaling. Cast_WRKY 31 should activate transcription of *LRR-RLK*. *Cast_Myb4* regulate SA accumulation via ABA suppression. HR could be activated by many mechanisms: SA or calcium signaling, via *Cast_Gnk2-like* (actin-dependent) or by vital protein degradation (by *Cast_RNF5*). Cell walls not infected may be reinforced and antifungal proteins may be secreted in more abundance, inhibiting further colonization. Triangles: *P. cinnamomi* PAMPs; Circles: Gnk2-like proteins.

In conclusion, a basal defense system may be acting in the response of *C. crenata* to *P. cinnamomi*. A lower and delayed expression of the eight studied genes was found in *C. sativa*, which may be related with the sensitivity of this species towards the disease. One probable explanation for this difference can be the allelic variation of the genes or gene-promoters that in *C. sativa* may condition the basal levels of gene expression. C. *mollissima*, also a resistant species, may share with *C. crenata* some of the allelic variants that allow an efficient level of resistance against *P. cinnamomi*. Natural selection could have had an active role in keeping those allelic variants, since Asian species have evolved in contact with *P. cinnamomi*. This study is part of an ongoing Portuguese breeding program to introduce resistance to *P. cinnamomi* in *C. sativa*. This knowledge may contribute for the development of strategies to control ink disease in chestnut and other woody plants, which may include early selection of resistant genotypes.

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Supplementary material

Supplementary material 1: Plant biological replicates obtained by *in vitro* culture. (A-D). *In vitro* micropropagation phases: (A) Establishment of an axillary node explant from the F1 mother plant; (B) Shoot multiplication; (C) Shoot elongation; (D) Pre-rooting in activated charcoal medium. (E-G) *Ex-vitro* phases: (E) *Ex-vitro* rooting; (F) Acclimatization, plantlets primary hardening; (G) Plantlets with 80 days after acclimatization used for inoculation.

Supplementary material 2. Primers and probe sequences and fluorophores of eight *Castanea crenata* (Japanese, resistant) candidate genes to *P. cinnamomi* resistance. Contig names like in Serrazina et al. 2015 and Chapter III.

Gene acronyms	Contig name	Primer forward sequence	Primer reverse sequence	Probe Sequence
Cast_Gnk2-like	CCI_CCN_005174	CACCACGACAAAGAGCAAGT	CCACCAATGACCCATATGAA	ACCAAAGCCCAGGAGAGG
Cast_PE-2	CCI_CCN_002220	TGACATCAACGGCAAGAGAT	AATGTCAAGTGCAGCCAAAC	CCAGGCCCGACACGTCTCAA
Cast_ABR1	CCI_CCN_001635	GATGTGGAGTCTCCCTGTGA	TCTGCTCCTGCTTTTGCTT	CGCCCCCTCTTTTGGCCA

Cast_C2CD	CCI_CCN_008363	CATGTGGAAGAGGAAACCT	GGAAAACTGAAATCAATTGAAG	ACGGTGGATGGAAACAGTCTGCA
Cast_LRR-RLK	CCI_CCN_000829	CAATTCTCGAAAGTTGAACGA	GCTTAGGACTCACCCAATGC	TCACCGGCCAATCTGCAATTG
Cast_Myb4	CCI_CCN_004144	TACAGCCCAATTTCCATTCA	CCAGCTCCAATGAAAAGGTT	TGGAACCAGACTATAGCGATGGCTCA
Cast_WRKY31	CCI_CCN_000812	GGTCTCTTCATCGGAAGGAA	ACAAGCCGCTCCTCACTAAT	ACGGTCAGGATCGCCCGGTA
Cast_RNF5	CCI_CCN_006887	GGATTCCGTCAGCGTACAG	AGCAGCTCATGTTCCGATAG	TGAAGAGGCTGCTTTTGCTTATCGC

Chapter V

Genetic mapping of resistance to *Phytophthora cinnamomi* in interspecific progenies of *Castanea* species

The work presented in this chapter was and will be published in the following research publications:

Santos C., Zhebentyayeva T., Serrazina S., Nelson C.D. and Costa R. (2015) Development and characterization of EST-SSR markers for mapping reaction to *Phytophthora cinnamomi* in *Castanea spp*. Scientia Horticulturae 194, 181-187. doi: 10.1016/j.scienta.2015.07.043

Santos C., Nelson C.D., Machado H., Gomes-Laranjo J., and Costa R. (2017) First interspecific genetic linkage map for *Castanea sativa* x *Castanea crenata* revealed QTLs for resistance to *Phytophthora cinnamomi*. Tree Genetics and Genomes (submitted)

In these research papers Carmen Santos participated in the experimental design, controlled crosses, SSR development, characterization and genotyping, phenotyping, map construction, QTL analyses and papers writing.

Abstract

The Japanese chestnut (*Castanea crenata*) carries resistance to *Phytophthora cinnamomi*, the destructive and widespread oomycete causing ink disease. The European chestnut (*Castanea sativa*), carrying little to no disease resistance, is currently threatened by the presence of the oomycete pathogen in forests, orchards and nurseries. Determining the genetic basis of *P. cinnamomi* resistance, for further selection of molecular markers and candidate genes, is a prominent issue for implementation of marker assisted selection in the breeding programs for resistance. In this study, quantitative trait loci (QTLs) for *P. cinnamomi* resistance were mapped on the first interspecific genetic linkage map constructed using two chestnut mapping populations, obtained by crossing *C. sativa* x *C. crenata*. Chestnut progenies were genotyped using 452 microsatellite and single nucleotide polymorphism molecular markers derived from the available chestnut transcriptomes. Forty-three of those microsatellite were developed from Expressed Sequence Tags (ESTs) differentially expressed *C. sativa* and *C. crenata*. These markers showed polymorphism and remarkably high interspecific transferability rate among chestnut species.

The consensus genetic map spans 714,8 cM and contains 283 markers mapped with an average interval of 2.5 cM. For QTL analyses, the progression rate of *P. cinnamomi* lesions in excised shoots inoculated was used as the phenotypic metric. Using non-parametric and composite interval mapping approaches, 10 QTLs were identified for disease resistance, distributed in five linkage groups: B, E, I, J and K. The presence of QTLs located in linkage group E regarding *P. cinnamomi* resistance is consistent with a previous preliminary study developed in American x Chinese chestnut populations, suggesting the presence of common *P. cinnamomi* defense mechanisms across species. Results presented here extend the genomic resources of *Castanea* genus providing potential tools to assist the ongoing and future chestnut breeding programs.

Keywords

Castanea spp., EST-SSR, SNP, genetic map, QTLs, *P. cinnamomi* resistance

Introduction

Chestnuts (Fagaceae family) are found around the world in the temperate zone, where they are very valued by different cultures for the nutritious nuts, valuable timber and landscaping purposes. European chestnut (*Castanea sativa*) produces the highest quality and most appreciated nuts, being a major economic income for the European mountainous producing regions, most of them are being threatned of depopulation. The emergence of heavily damaging diseases, namely ink disease and chestnut blight, caused by *Phytophthora cinnamomi* and *Chryphonectria parasitica*, respectively are responsible for the decline of the European production from 430,000 tones to 140,000 tones in the past 50 years.

P. cinnamomi is one among the most destructive pathogens associated with the decline of forestry, ornamental and fruit species (Hardham 2005; Kamoun et al. 2014). When introduced into an environment, this pathogen has enormous impacts on natural systems, and has been shown to reduce the native biodiversity in Europe, the USA, Australia, New Zealand and Africa. In Portugal ink disease has become widespread since *P. cinnamomi* was first recorded in 1838. In North America, prior to the decimation of the American chestnut (C. *dentata*) by chestnut blight, ink disease was partially responsible for its decline in the southeastern part of its range (Anagnostakis 2001). Although *C. sativa* and *C. dentata* are susceptible to ink disease and chestnut blight, the Japanese and Chinese chestnuts (*C. crenata* and *C. mollissima*, respectively) show resistance to both diseases (Crandall et al. 1945). Taking profit of this fact, The American Chestnut Foundation (TACF) have pursued a backcross breeding program, using *C. mollissima* as donor parents of resistance, to introgress resistance to *C. parasitica* into American

chestnut. Nevertheless, ink disease is currently re-emerging in the USA and constitutes a serious threat to the American chestnut restoration program (Jacobs et al. 2013). Consequently, breeding for resistance to *P. cinnamomi* was recently initiated in USA (Jeffers et al. 2009; Zhebentyayeva et al. 2014). In Portugal, a chestnut breeding program was initiated in 2006 aiming to introgress the resistance from *C. crenata* into *C. sativa*, by crossing both species. Hybrid progenies, segregating for *P. cinnamomi* resistance, have been obtained and extensively studied in order to understand the chestnut resistance mechanisms to ink disease. Accurate phenotyping methodologies were developed to score diverse metrics of resistance of each progeny aiming to identify marker:trait associations (Santos et al. 2015). Moreover, a group of hybrid genotypes selected as the most resistant to *P. cinnamomi*, are being propagated as improved genetic materials for new rootstocks release to the market in the near future. Additionally, root transcriptomes of *C. sativa* and *C. crenata* inoculated and non-inoculated with *P. cinnamomi* were sequenced (Serrazina et al. 2015, Chapter III), constituting an important increase in available genomic resources that provide an advantage for the identification of candidate genes for ink disease resistance.

Despite the economic and ecological importance of woody plants, mapping of QTLs for important phenotypic traits have been rather limited on these species when compared with major crops or model plants. For chestnut, extensive genomic resources have been developed mainly for *C*. *mollissima* and *C. dentata* (Kubisiak et al. 1997; Sisco et al. 2005; Fang et al. 2013). Kubisiak et al. (1997) published the first genetic map based on a F2 population of an interspecific cross of *C. dentata* and *C. mollissima*, where three QTLs for blight resistance were proposed. In 2005, Sisco et al. presented an updated version of the map adding 304 markers. Recently, a new genetic map of *C. mollissima* was created using 1393 new markers (developed from *C. mollissima* and *C. dentata* transcriptome datasets), constituting the reference map in which the physical map was anchored

(Barakat et al. 2009; Barakat et al. 2012; Kubisiak et al. 2013; Fang et al. 2013).

Nevertheless, the availability of molecular markers, genetic maps and QTLs' identification is limited for other *Castanea* species. So far, only one genetic map was released for *C. sativa*, based on a F1 full-sib population of 96 trees, which was used for mapping QTLs for adaptive traits (Casasoli et al. 2001; Casasoli et al. 2004). Regarding to molecular markers development, small sets of SSR markers were developed from enriched genomic libraries of European chestnut (Marinoni et al. 2003; Buck et al. 2003) and Japanese chestnut (Yamamoto et al. 2003; Inoue et al. 2009). More recently, a large set of EST-SSRs were developed from Japanese chestnut (Nishio et al. 2011). However, the development of EST-SSRs from datasets targeting a specific phenotypic trait, such as *P. cinnamomi* resistance, has not been addressed in chestnut so far.

In the present study, the first European x Japanese chestnut genetic map was constructed using markers developed from *C. mollissima*, *C. dentata*, *C. sativa* and *C. crenata* transcriptomes. Markers developed from *C. sativa* and *C. crenata* were also characterized for polymorphism and transferability among four *Castanea* species. Additionally, QTLs for *P. cinnamomi* resistance were detected and mapped for the first time in *Castanea* populations, which is a major breakthrough on a tree which the genome has not yet been sequenced. These new genomic resources should be useful mainly in Europe and in the USA, aiming at improving resistance to ink disease or even for another woody species threaten by *P. cinnamomi* such as oaks, *Quercus* spp.

Materials and Methods

Plant material

Genetic map construction and QTL mapping were carried out using two full-sib families of *C. sativa* (female) x *C. crenata* (male), obtained from controlled

pollination. Genotypic and phenotypic data were collected for 74 progenies from *C. sativa* (cultivar Aveleira- CSAV) x *C. crenata*2 cross (SC) and 81 progenies from *C. sativa* (cultivar Bária- CSBA) x *C. crenata*1 cross (BC).

Table 1. Twenty-five chestnut samples were used to characterize the candidate EST-SSR markers, and representatives of four *Castanea* species were used to investigate cross-species transferability. TACF: The American Chestnut Foundation.

Progenitor Tree	Species	Origin
CSAV	European chestnut	Portugal
CSBA	European chestnut	Portugal
CSDE	European chestnut	Portugal
CSMT	European chestnut	Portugal
CSSOU	European chestnut	Portugal
CSTRI	European chestnut	Portugal
CSVER	European chestnut	Portugal
C. crenata1	Japanese chestnut	Portugal
C. crenata2	Japanese chestnut	Portugal
C. crenata3	Japanese chestnut	Portugal
Cranberry	American chestnut	TACF, USA
AD98	American chestnut	TACF, USA
NCDOT	American chestnut	TACF, USA
Pryor-79	American chestnut	TACF, USA
Pryor-180	American chestnut	TACF, USA
Ted Farm A	American chestnut	TACF, USA
Mahogany	Chinese chestnut	TACF, USA
Vanuxem	Chinese chestnut	TACF, USA
Nanking	Chinese chestnut	TACF, USA
Cataloochee-70	Japanese chestnut *x* American chestnut (F1)	TACF, USA
Adair KY115	Chinese chestnut (Mahogany) x American chestnut (F1)	TACF, USA
JB197	Chinese chestnut *(Mahogany) x* American chestnut (BC2)	TACF, USA
Cliffs GL-5	Chinese chestnut *(Nanking) x* American chestnut (F1)	TACF, USA
Cliffs GL-61	Chinese chestnut *(Nanking) x* American chestnut (F1)	TACF, USA
WWC70	Chinese chestnut *(Nanking) x* American chestnut (F1)	TACF, USA

From the SC cross, 22 individuals did not show alleles from the male parent and therefore only the alleles from *C. sativa* female parent were considered for marker segregation analysis (haploid population type).

For SSR development, primer pairs were characterized with a test set of 25 individual trees. Specifically, the test set consisted of 7 European and 3 Japanese chestnuts from the Portuguese breeding program; 6 American, 3 Chinese used in TACF's breeding program, and 5 Chinese x American and 1 Japanese x American F1 hybrid trees used as parents in BC1 crosses by TACF (Table 1).

Molecular markers source and development

A total of 2974 transcriptome-derived molecular markers developed from *C. mollissima*, C. *dentata*, *C. sativa* and *C. crenata* (421 simple sequence repeats-SSRs and 2553 single nucleotide polymorphism-SNPs) were used to test polymorphism parental genotypes (*C. sativa* and *C. crenata*).

The DEGs identified in root transcriptomes of *C. crenata* and *C. csativa* inoculated and non-inoculated with *P. cinnamomi* were used as sources of ESTs for development of new SSRs (see details in Serrazina et al. 2015 and Chapter III). Candidate SSR sequences were located and primer pairs were designed with the SSR Locator program (Maia et al. 2008). The criteria used for locating candidates were SSR motifs with 14 or more repeat sequences: that is, >6 repeats of di-nucleotide motifs, >5 repeats of tri-nucleotide motifs, >4 repeats of tetra-nucleotide motifs and >3 repeats of penta-nucleotide motifs.

Primer selection parameters were set for 99 SSRs previously found: a product size range of 100-500 base pairs (bp); a primer size of 18-25 bp (with an optimum of 20 bp); a primer Tm of 55-65ºC (optimum of 60ºC); and an optimum primer GC content of 50% with a minimum of 35%. A set of 60 primer pairs SSRs identified by the SSR Locator were then filtered by the following criteria: exclusion of redundant primer pairs identified in both DEGs

sources; and exclusion of primers with inappropriate patterns, such as: low GC content (<35%) at the 5' and 3' ends of primer sequences or irregular nucleotide distribution, such as SSR pattern. A finalized list of 58 SSRs was selected for PCR amplification test. The SSR names were associated to chestnut species using the prefix Cs or Cc indicating respectively *C. sativa* or *C. crenata* candidate genes associated with resistance to *P. cinnamomi*, followed by PT (Portugal) and a four-digit number identifier (CsPT0001-CsPT0023 and CcPT0001-CcPT0035).

The molecular markers from *C. mollissima* and *C. dentata* used in this study, were previously developed by Kubisiak et al. (2013): 378 SSRs from *C. mollissima* (named by CmSI*number*) and SNP markers from C. *mollissima* CCall_Unigene_V2 assembly data and from *C. dentata* AC454_Unigene_V3 contig data (www.fagaceae.org). SNPs were called as CCallv2contig number_SNP position and AC454v3contig number_SNP position, respectively (Kubisiak et al. 2013). In this study, in order to simplify the nomenclature, they were named as CC_*contig_number* SNP position and AC_*contig_number* SNP position, respectively. From those SNPs developed from *C. mollissima*, 1536 were used for chestnut reference genetic map and were denominated as CmSNP00001-CmSNP01536 (Supplementary material 1, Kubisiak et al. 2013). This nomenclature was kept for common SNPs used in this study and in *C. mollissima* reference genetic map. When available, linkage group (LG) information from reference map (A to L) was placed in front of marker name.

<u>New SSRs amplification and characterization</u>

Total genomic DNA was extracted from 100 mg of fresh leaves using the DNeasy™ Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions.

New SSR marker analysis was facilitated using a M13-specific sequence (5'-CACGACGTTGTAAAACGAC-3'), which was added to the 5' end of each

forward primer (Schuelke, 2000). To favor 3' adenylation of the forward amplified strand, a PIG-tail (5'-3') was added to 5' end of each reverse primer (Brownstein et al. 1996). For fluorescent detection, PCR using three primers was performed: forward and reverse primers and a 5′ dye (6-FAM, NED, PET or VIC)-labeled M13-specific primer (same sequence as the M13 'tail' described above). Loci were amplified individually in 12.5 µl reaction containing: 20 ng of template DNA, 0.16 µM of 5'-dye-labeled M13 primer, 0.04 µM of 5´-tailed forward primer, 0.16 µM of reverse PIG-tailed primer, 66 µM of dNTPs, 2 mM of $MgCl_2$, 2 µl 5x GoTaq Flexi Buffer (Promega) and 1.0 U of GoTaq® Flexi DNA Polymerase (Promega). Amplifications were undertaken in a Biometra® T1 Thermocycler using the following profile: 94°C for 2 min; 94°C for 30 s, 60°C (annealing temperature for primer forward and reverse) for 45 s, 72ºC for 45 s during 30 cycles; 94°C for 30 s, 53°C (annealing temperature for 5'-dye-labeled M13 primer) for 45 s, 72ºC for 45 s during 8 cycles; 72 °C for 10 min; indefinite hold at 4ºC. Completed reactions were loaded onto an ABI PRISM 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA) and run according to the manufacturer's protocol. Allele sizes were determined using the ROX500 internal size standard and the global southern algorithm implemented by ABI PRISM GeneMapper software version 4.0 (Applied Biosystems). Alleles were named according to Deemer and Nelson (2010) using the European and Japanese chestnut as reference samples and alleles.

The observed heterozygosity (H_O) and expected heterozygosity (H_e) were calculated for all SSRs that amplified a unique fragment using GeneAlEx software v. 6.5 (Peakall and Smouse, 2012). H_O was calculated as the number of heterozygous individuals divided by the total number of individuals. H_e was calculated using an unbiased formula from allele frequencies as $1 - \sum_i^2 p$ ($1 \leq I \leq m$), where *m* is the number of alleles at the target locus and *pi* is the allele frequency of the *i*th allele at the target locus.

Genotyping of *C. sativa* x *C. crenata* populations

From 2974 markers used for genotyping parental individuals, 452 of them (180 SSRs and 272 SNPs) showed polymorphism, at least, for one parent, and therefore they were used for genotyping all progenies.

The method reported in the previous section was used for SSR genotyping. GoldenGate BeadArray platform and GenomeStudio software were employed for SNP detection, clustering, genotype calling and confidence scores assignment. However, data for all SNPs were inspected manually and edited if necessary (Yan et al. 2010). SNP genotyping was only performed for 26 genotypes from the SC cross.

Linkage map construction

Linkage and marker distortion analyses and map construction were performed using JoinMap v.3.0 software (van Ooijen 2001). Maps were first constructed separately for both mapping families: SC and BC, using CP (Cross Pollination) model. Also, a *C. sativa* female parent (cultivar Aveleira) map was constructed using 74 individuals, using HAP (haploid type) population model. Linkage Groups (LGs) were determined at a logarithm of the odds (LOD score) with a minimum threshold of 4.0. Linkage maps were calculated using the Kosambi mapping function (Kosambi 1944) and applying default mapping parameters: all linkage recombination estimates smaller than 0.4 and a LOD larger than 1.0. Syntenic groups among the three maps were identified and combined through the JoinMap 'Combine Groups for Map Integration' function. Map orientation was assigned by comparison with C. *mollissima* reference map (Kubisiak et al. 2013). Linkage groups were drawn using MapChart 2.0 software (Voorrips 2002).

QTL mapping

QTL analyses were performed using MapQTL 5.0 software (Van Ooijen 2004). Utilizing the consensus genetic map created here, QTLs for *P.*

cinnamomi resistance were detected for SC population and also for *C. sativa* female parent (using haploid model- HAP_{SC}). Progression rate of *P. cinnamomi* lesions in inoculated excised shoots was chosen as phenotypic metric as it provided the most differentiation among progenies. To obtain this metric, an average of 7.7 excised shoots from each progeny was inoculated with *P. cinnamomi*, in two different seasons (spring and autumn). The external lesion length was measured at five time points after inoculation and the lesion progression rates (cm/day) were calculated for each individual, across both seasons (Santos et al. 2015, Chapter II).

Marker significance level was estimated using the Kruskal-Wallis analysis (K-W, non-parametric test); and the interval mapping method was used to detect the presence of a putative QTL (Jansen and Stam 1994). Then, cofactors significantly associated to the trait ($P < 0.02$) for Multiple QTL model (MQM) computation, were estimated by applying a backward elimination procedure ('Automatic cofactor Selection' MapQTL function) through selection of the closest markers to the QTL peak. The significant LOD threshold ($P < 0.05$) was estimated from 1 000 permutations of the phenotypic trait, for each LG. QTLs were declared when LOD score (MQM) exceeded the minimum significance threshold.

The R^2 value, representing the percentage of the phenotypic variance explained by the marker genotype at the QTL, was taken from the peak QTL position as estimated by MapQTL software.

Allelic effects for each parent were calculated using the estimated phenotypic means associated to each of the four possible genotypic classes ac, ad, bc, and bd, deriving from a CP segregating and given by the software. Female (*C. sativa*) additivity was calculated as $ACs=(\mu_{ac}+\mu_{ad})-(\mu_{bc}+\mu_{bd})/4$ and male (*C. crenata*) additivity as $ACc=(\mu_{ac}+\mu_{bc})-(\mu_{ad}+\mu_{bd})/4$ (Ben Sadok et al. 2013). Negative values indicate that the alleles increased resistance trait, since the phenotypic data mesuared susceptibility (lesion progression rate after *P. cinnamomi* inoculation) level.

Each significant QTL was characterized by the peak marker and the other associated markers within the QTL region, the percentage of phenotypic variation explained (R^2), LOD score, K-W test, effects of the alleles for each parent and the LOD-1 confidence region. QTL representations were drawn using MapChart 2.0 software (Voorrips 2002).

As the molecular markers were developed from transcriptome sequences, putative resistance genes within the QTL intervals were identified through BLASTn query against the NCBI database.

Results

SSR markers development and characterization

A total of 99 SSRs, meeting our SSRs searching criteria, were found in the EST sequences of *P. cinnamomi* resistance candidate genes, in which 43 were found in the 305 *C. sativa* sequences and 56 in the 283 *C. crenata* sequences. Among them, a total of 60 SSR sequences were useful for primer design and after filtering as described, 58 SSRs were selected for PCR testing and further characterization (Table 2).

Table 2. Summary of the number of EST-SSRs found and selected using the SSR Locator software. Total number of SSRs corresponding to all candidate SSRs meeting SSR searching criteria; Total number of SSRs with primers means SSRs where appropriate primers where found; Total number of SSRs selected corresponding to the final set of SSRs for testing amplification and polymorphism.

Source of candidate genes	Total number of SSRs	Total number of SSRs with primers	Total number of SSRs selected
C. sativa	43	24	23
C. crenata	56	36	35
Total	99	60	58

Of tested 58 SSRs, 43 (74.1%) gave unique amplification products about the predicted target size with DNA samples of at least 3 progenitor-trees, whereas 15 primer pairs showed no amplification or non-specific fragment amplification. The percentage of success in PCR amplification was similar for *C. sativa* (72.7%) and *C. crenata* (77.1%). 43 and 41 (95.4%) SSRs showed strong and selective amplification in European chestnut and in Japanese chestnut, respectively. Regarding the parental trees from the American breeding program, almost all SSRs (97.7%) amplified in American chestnut and 90.7% amplified in Chinese chestnut individuals. Thirty-four SSRs (79.1%) amplified from DNA of four chestnut species, while twenty-five SSRs (58.1%) were found to amplify in all 25 samples (representing the four tested species and two types of hybrids). SSRs with di-, tri-, and tetra-nucleotide motifs showed no distinct differences in transferability.

The polymorphism of each locus was evaluated by the number of alleles and expected and observed heterozygosity. SSRs characterization results are summarized in Supplementary material 2. For all primer pairs, an average of 5.26 ± 0.39 alleles were obtained for the individuals tested. Three SSR markers (CcPT_0002, CcPT_0019 and CcPT_0026) were monomorphic. The number of alleles obtained for the polymorphic SSRs ranged from 2 (CcPT_0001, CsPT_0016 and CsPT_0017) to 11 (CcPT_0013). The observed and expected mean heterozygosity was 0.39 ± 0.03 and 0.61± 0.04 respectively. Marker CcPT_0020 presented the highest observed heterozygosity (0.82) while the marker CcPT_0024 showed the lowest observed heterozygosity (0.043). The expected heterozygosity ranged between 0.22 (CsPT_0017) and 0.87 (CsPT_0003).

The most abundant repeat type was the tri-nucleotide (65.1%) followed by the di-nucleotide (27.9%). There was a predominance of CT/AG and TC/GA motifs among di-nucleotide repeats and TTG/AAC and CTT/AGG for tri-nucleotide repeats. As indicated, a higher number of alleles and higher heterozygosity values, di-nucleotide SSRs are slightly more polymorphic,

compared with the tri- and tetra-nucleotide SSRs (Supplementary material 2).

Construction and analysis of genetic linkage map

From the 2972 molecular markers (419 SSRs and 2553 SNPs) screened with the *C. sativa* and *C. crenata* parents, 435 markers (163 SSRs and 272 SNPs) were polymorphic for the SC population; and 92 SSRs revealed polymorphism in the BC population (no SNPs genotyping was performed for this population). The consensus map consists of 283 markers (132 SSRs and 151 SNPs) mapped to 15 LGs (Figure 1). According to the genetic reference map (Kubisiak et al. 2013), all molecular markers developed from *C. mollissima* (CmSInumber and CmSNPnumber) were correctly mapped on each LG. However, marker positions are not in the same order (chestnut reference map is in Supplementary material 1). A total of 37 SNPs mapped in the C. *mollissima* reference map were also integrated in the present consensus map. The number of LGs (15) is not the expected (n=12). Three LGs were not completely joined, containing markers assigned for the same LG (in reference map) in two different groups: LG_E, LG_H and LG_J. Consequently, those unlinked LGs were named as LG_E/LG_E1, LG_H/LG_H1 and LGJ_/LG_J1, respectively, and were placed in sequence in the map graphic (Figure 1).

The interspecific genetic map spans a total genetic distance of 714.8 cM (96.3% of genetic distance in the C. *mollissima* reference map) with an average interval of 2.5 cM between markers. The genetic length of the linkage groups ranged in size from 18.5 (LG_L) to 87.8 cM (LG_A), with an average length of 47.7 cM. From all markers mapped, nineteen (6.7%) of them exhibited poor goodness-of-fit (X^2 values > 5.0), nonetheless they were kept, since the majority were in accordance with the reference map. High distortion was observed for the unmapped molecular markers.

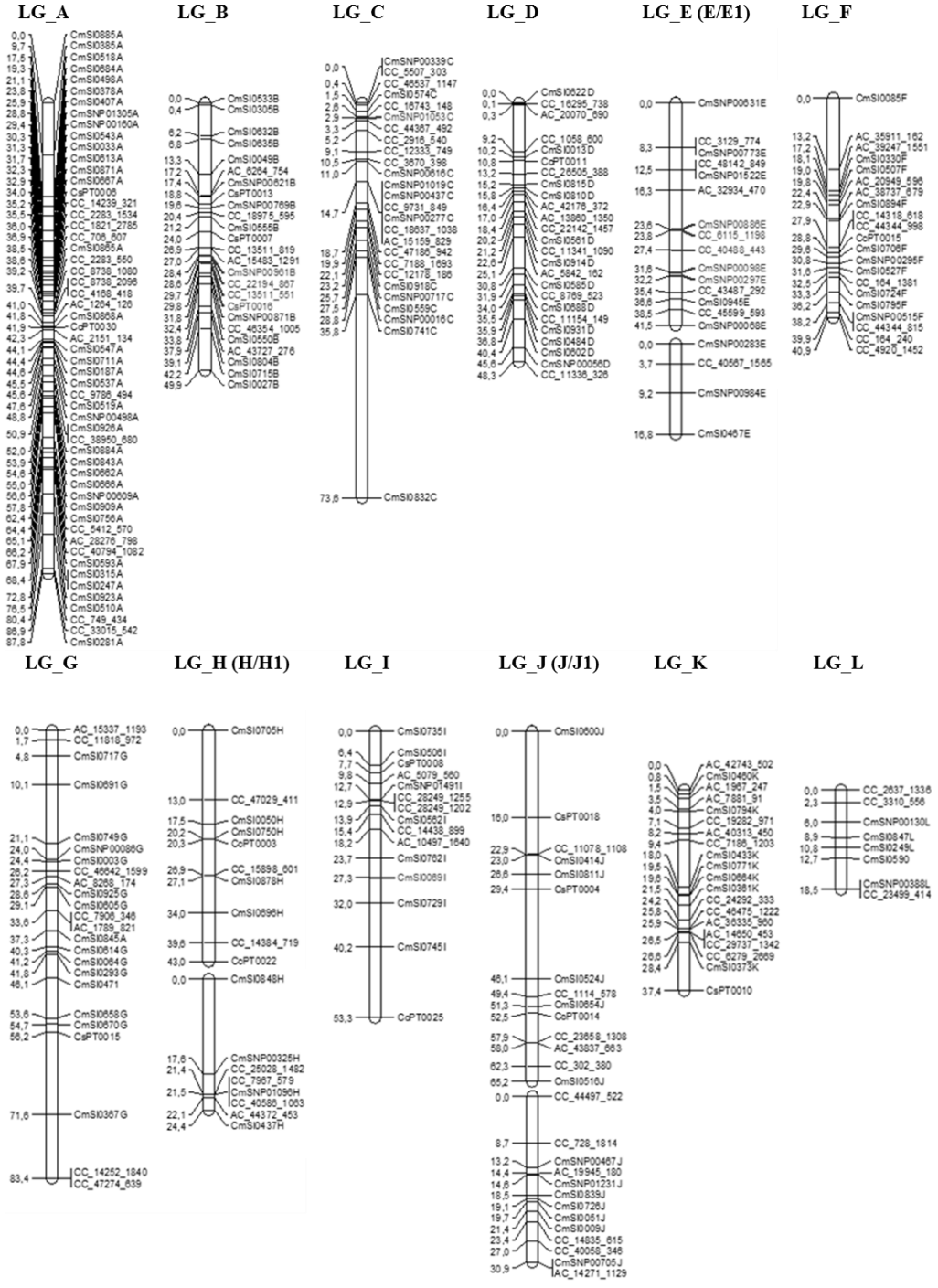

Figure 1. *C. sativa* x *C. crenata* interspecific consensus genetic map containing 283 markers (SSRs and SNPs) mapped on fifteen linkage groups (LGs). Marker

positions on LGs were assigned to the *C. mollissima* reference map LGs (Kubisiak et al. 2013), and those that are not joined (LG_E/LG_E1, LG_H/LG_H1 and LGJ_/LG_J1) were placed in sequence.

QTL mapping

QTL analyses for the resistance to *P. cinnamomi* was performed for SC population and for *C. sativa* female parent, in an haplod model; nevertheless, only SC population revealed loci with significant effects (K-W test, $P<0.05$) and LOD scores above the estimated threshold. For SC population, ten QTLs were identified in five LGs: LG_B, LG_E, LG_I, LG_J and LG_K and therefore each QTL interval was named as *Pc*_B(1-3), *Pc*_E(1-3), *Pc*_I1, *Pc*_J(1-2) and *Pc*_K1 (Table 3 and Figure 2). The percentage of the phenotypic variance explained by the QTLs varied from 5.7 to 33.3% and resulted mainly from *C. crenata* additive effect (Table 3). QTL intervals were localized to regions less than 11 cM on the consensus map, the largest being *Pc*_E1 covering 10.3 cM. Fourteen molecular markers (3 SSRs and 11 SNPs) were associated to the QTLs revealed (Table 3 and Figure 2, underlined). The strongest QTLs (based on LOD scores and K-W test) were identified in LG_E (*Pc*_E1, *Pc*_E2 and *Pc*_E3), with *Pc_E1* showing the highest significance levels in K-W test ($\alpha=0.005$ to 0.001). The QTLs of LG_E resulted from *C. crenata* additive effects (*Pc*_E1) or from both parents' additive effects (*Pc*_E2 and *Pc*_E3) (Table 3 and Figure 2).

In LG_B three QTLs were mapped (*Pc*_B1, *Pc*_B2 and *Pc*_B3), in which *Pc*_B2 was the strongest, showing the highest LOD scores and explaining 33.3% of phenotypic variation resulting from a *C. sativa* additive effect. In contrast, the *Pc*_B1 and *Pc*_B3 QTLs resulted from *C. crenata* additive effects (Table 3 and Figure 2).

Two QTLs were identified in LG_J, however the LOD-1 confidence interval shared the same genomic region, suggesting a single QTL existing in the lower part of LG_J.

Table 3. Results of MQM mapping for QTLs conferring resistance to *P. cinnamomi* in *C. sativa* x *C. crenata* population. LOD score, position in the LG, Kruskal-Wallis test and additive effect are presented for the marker at the LOD peak and for the other associated markers.

QTL	LG	Interval (cM)	Interval 1-LOD (cM)	R^{2a} (%)	Peak marker					Associated loci				
					Marker	LOD score	Position (cM)[b]	K-W test[c]	Additive effect[d]	Marker	LOD score	Position (cM)[b]	K-W test[c]	Additive effect[d]
*Pc*_B1	B	19.6-20.4	18.8-20.4	17.1	CmSNP00769B	3.61	19.6	**	A_{Cc} (-0.02)	-	-	-	-	-
*Pc*_B2	B	29.8-31.8	29.8-31.8	33.3	CsPT0016	4.32	29.8	**	A_{Cs} (-0.06)	CmSNP00871B	4.24	31.8	**	A_{Cs} (-0.06)
*Pc*_B3	B	45.2-49.9	42.2-49.9	18	CmSI0027B	4.64	49.9	-	A_{Cc} (-0.10)	-	-	-	-	-
*Pc*_E1	E	0.0-10.3	0.0-10.3	16.5	CC_3129_774	3.95	8.4	*****	A_{Cc} (-0.13)	CmSNP00773E	3.95	8.4	*****	A_{Cc} (-0.13)
										CmSNP00631E	3.87	0.0	****	A_{Cc} (-0.07)
*Pc*_E2	E	21.4-31.4	21.4-31.4	17.8	CmSNP00886	4.96	23.6	**	A_{Cc} (-0.03)	CC_6115_1198	3.97	23.9	***	A_{Cc} (-0.07) A_{Cs} (-0.08)
										CC_40488_443	4.62	27.4	**	A_{Cc} (-0.09) A_{Cs} (-0.04)

QTL	LG	Interval (cM)	Interval 1-LOD (cM)	R^{2a} (%)	Peak marker					Associated loci				
					Marker	LOD score	Position (cM)[b]	K-W test[c]	Additive effect[d]	Marker	LOD score	Position (cM)[b]	K-W test[c]	Additive effect[d]
*Pc*_E3	E	35.2-35.4	35.2-35.4	17.8	CC_43487_292	4.97	35.4	-	A_{Cc} (-0.09) A_{Cs} (-0.65)	-	-	-	-	-
*Pc*_I1	I	40.2-48.2	44.2-51.5	20.1	-	3.89	46.2	-	A_{Cs} (-0.16)	-	-	-	-	-
*Pc*_J1	J	25.4-27	24.4-30.9	5.7	-	3.43	24.4	-	-	-	-	-	-	
*Pc*_J2	J	29-30.9	24.4-30.9	9.1	CC_33657_1284	2.47	30.9	***	-	AC_14271_1129	2.47	30.9	***	-
*Pc*_K1	K	32.4-37.5	32.4-37.5	16.2	CsPT0010	4.77	37.5	-	A_{Cc} (-0.04)	-	-	-	-	-

[a] Percent explained phenotypic variance
[b] QTL position in cM from the top of the Linkage Group
[c] Kruskal-Wallis test: *=0.1, **=0.05, ***=0.01, ****=0.005 and *****=0.001.
[d] Additive effect, A_{Cs}= additive effect from *C. sativa* and A_{Cc}= additive effect from *C. crenata*. Negative values indicate that the alleles increased resistance trait value.

All markers found to be associated to QTL belong to transcribed sequences. CsPT0016 (*Pc*_B2) and CsPT0010 (*Pc*_K1) are SSR found in the sequences of EST of differentially expressed genes after inoculation with *P. cinnamomi* in *C. sativa*. CsPT0016 is a SSR found in an EST annotated as a Pathogenesis-related transcriptional factor/Ethylene responsive transcription factor (ERF), while the SSR CsPT0010 was found in an EST annotated as containing a stress-associated protein domain belonging to the Zinc-finger family. SNPs associated with identified QTLs were markers of genes involved in diverse cellular processes: synthesis of aromatic compounds (CmSNP00769B), hormone signaling (CC_3129_774) and regulation of gene expression (CmSNP00773E and CmSNP00631E). The remaining SNPs marking QTLs were developed from genes with uncharacterized functions to date. Finally, CmSI0027B SSR maker was identified in a high mobility group B protein transcript, which may modulate different nuclear processes upon exposure to stress (Antosch et al. 2012).

Discussion

Ten QTLs associated with resistance to *P. cinnamomi* were identified for the first time in a *C. sativa* x *C. crenata* reference population, providing an improved understanding about the genetic architecture of pathogen resistance in tree species. QTLs were mapped in a new chestnut interspecific genetic map constructed with functional molecular markers located in transcribed sequences (transcriptome-derived). Some of those markers were developed in this study from DEGs after *P. cinnamomi* inoculation. Nevertheless, the majority of the mapped markers are shared with the *C. mollissima* reference map (Kubisiak et al. 2013), which enabled to identify the correspondent LGs, increasing the robustness of the obtained *C. sativa* x *C. crenata* map.

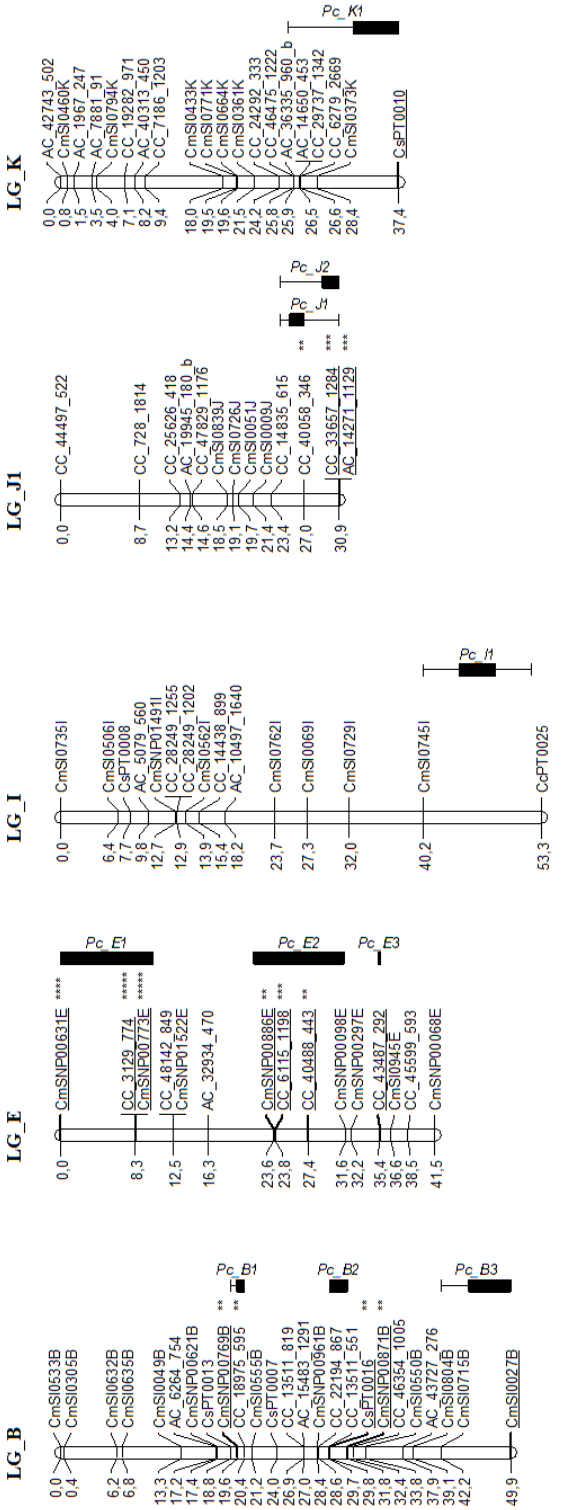

Figure 2. Genomic positions of QTLs detected on the linkage groups of the *C. sativa* x *C. crenata* consensus genetic map by multiple QTL mapping (MQM) for *P. cinnamomi* resistance. QTLs are represented by black boxes extended by lines representing the LOD-1 confidence interval. Markers associated with the QTL intervals are underlined. Asterisks represents the significance levels obtained by Kruskal-Wallis test for marker association to the trait: *=0.1, **=0.05, ***=0.01, ****=0.005 and *****=0.001.

In addition, each of the markers used was mapped to the same linkage group either on the reference map or on the map of *C. sativa* x *C. crenata*, providing evidence of strong conservation across *Castanea* species.

For the first time in chestnut, EST-SSR markers obtained from a set of DEGs identified after *P. cinnamomi* inoculation were used to obtain a genetic map. Stringent parameters were applied in order to select SSRs with specific amplification and high polymorphism rates. Consequently, 43 novel EST-SSR markers were developed and characterized. The success rate of amplification was 74.1% which is consistent with previous studies in Japanese and Chinese chestnut (Kubisiak et al. 2013; Nishio et al. 2011a).

Regarding the EST-SSRs set presented, 25 were transferable among the four chestnut species, using a set of 25 different individuals from the American and Portuguese chestnut breeding programs. In general, high transferability was observed among *Castanea* spp. ranging from 90.7% for Chinese chestnut and 100% for European chestnut. Similar observations were made in other studies in chestnut (Nishio et al 2011b; Kubisiak et al 2013) and in oak (Bodénès et al. 2012; Durand et al. 2010).

Combining two full-sib populations from unrelated parents for genetic mapping constitutes a strategy to increase genetic diversity, since native alleles from different species are sampled. The reported genetic map contains 283 molecular markers mapped in 15 LGs, achieving a very high coverage (714.8/742.4 cM), with a marker density 1 marker/2.5 cM, in average). In *Quercus robur* and *Q. petraea* genetic maps, a similar average of marker density was obtained: 1 marker/2.9 and 2.7 cM, respectively (Durand et al. 2010, Bodénès et al. 2012). In chestnut, Casasoli et al. (2001) achieved a *C. sativa* genetic map covering 720 cM, and an average of marker density of about 1 marker/ 9 cM was obtained. A higher density map was expected in this study, since from the 2972 molecular markers available, 452 were polymorphic and used for genotyping. The polymorphism rate obtained for *C. sativa* x *C. crenata* population was similar to *C. mollissima* population

used for constructing the reference map, indicating high transferability of markers from *C. mollissima* and *C. dentata* to *C. sativa* and *C. crenata* (Kubisiak et al. 2013). Nevertheless, about 37% of the polymorphic markers were not joined to the obtained LGs and therefore were excluded from the consensus map. Several reasons may be presented to explain the number of unmapped markers: 1) a low number of individuals per population; 2) missing values, mainly for SNP data, which were collected only for 26 SC individuals; 3) marker distortion and 4) absence of segregating markers in some genomic regions, avoiding strong linkages between markers with distant positions. The last hypothesis, could also explain the observation of three extra LGs relative to the expected (n=12). Concerning the distorted segregation, it appears to be common in interspecific crosses between woody plants. Incorrect paring of homologous chromosomes may occur during meiosis, resulting in segregation distortion. In this study, unmapped markers showed high distortion, which explains their exclusion from the consensus map. On the other hand, only 6.7% of the mapped markers showed distortion, which is a lower rate when compared with other studies of inter- or intraspecific crosses in *Castanea* and another woody species (Kubisiak et al. 1997; Brondani et al. 2006; Lowe and Walker 2006; Zhang et al. 2012). Accordingly, in *C. sativa* genetic map, 10% of the markers showed distortion, however the molecular markers used in that study were RAPD, ISSR and isoenzymes. Indeed, the consensus genetic map constructed for *Quercus* spp. with EST-SSRs showed only 0.9 to 6.8% of distorted markers, depending on the populations (Bodénès et al. 2012). The low rate of distorted markers suggests low presence of chromosome translocations from parents to the progenies, however cytogenetic studies are needed to confirm this supposition.

P. cinnamomi susceptibility was previously evaluated for each SC progeny, using diverse and accurate methodologies (Santos et al. 2015 and Chapter II). Due to the recalcitrance of the species which imposes limitations for *in*

vitro establishment and stem cuttings propagation to produce clonal individuals from each progeny, only 16 SC progenies were able to be phenotyped using the root inoculation test. In the present study, lesion progression rate in excised shoots inoculated with the pathogen was used as phenotypic metric for QTL analyses, because: i) we could have stems from all progenies; ii) lsion progression rates followed a continuous variation, from 0.15 to 1.13 cm/day, depending on the genotype, enabling the analysis of the trait in a quantitative manner and iii) the results from excised shoot inoculation method were strongly and negatively correlated (-0.85) with the days of survival in root inoculation assays, performed for a subset of SC progeny (Santos et al. 2015 and Chapter II).

QTL analyses were also performed for HAP-type population (HAP_{SxC}): using segregation data from the susceptible *C. sativa* female parent. No significant QTLs were detected contrasting with the ten QTLs identified in the hybrid SC population. Results suggest that *loci* or allelic variants conferring resistance were inherited from resistant male parent (*C. crenata*) and were lacking or defective in the susceptible *C. sativa*. Nevertheless, additive effects from *C. sativa* were obtained in some QTLs identified in SC population, indicating that heterozygous genotypes of *C. sativa* used in this study could have some allelic variants favorable to *P. cinnamomi* resistance.

Significant QTLs were detected in five LGs: LG_B, LG_E, LG_I, LG_J and LG_K. The *P. cinnamomi* resistance QTLs mapped on LG_E were the strongest in the *C. sativa* x *C. crenata* population, since the associated markers were highly significant associated to the trait and showed the highest LOD scores. Accordingly, *P. cinnamomi* resistance QTLs on LG_E were previously reported in a pilot study using two chestnut backcross families (*C. mollissima* x *C. dentata* hybrid) (Zhebentyayeva et al. 2014), suggesting a crucial role of those genomic regions in *P. cinnamomi* resistance. Moreover, new QTL analyses were performed for recent backcross families obtained in 2014 by The American Chestnut Foundation,

revealing QTLs for *P. cinnamomi* resistance again on LG_E and also in LG_K (Tatyana Zhebentyayeva, personal communication). QTL results obtained for American x Chinese chestnut crosses and European x Japanese chestnut crosses show consistency, indicating that those parents might share resistant haplotypes, located on LG_K and LG_E. Furthermore, the *Pc_B3* QTL, localized in 45.2-49.9 cM on LG_B seems to be in the same region of *Cbr1* QTL, conferring resistance to blight disease, which is located from 40.9 to 50.4 cM on LG_B in the chestnut reference map (Kubisiak et al. 1997, Kubisiak et al. 2013, Fang et al. 2013). This *Cbr1* QTL from chestnut is syntenic to a significant portion of scaffold 6 on peach genome, where QTLs for resistance to powdery mildew disease (*Sphaerotheca pannosa* var. *persicae*) have been identified (Foulongne et al. 2003, Staton et al. 2015). These findings suggest common resistance mechanisms to different diseases caused by fungal or fungal-like pathogens across *Castanea* genus and in the close relative (*Prunus* spp.) (Staton et al. 2015).

From the ten QTLs identified, five associated SNPs were exclusively mapped for *C. sativa* x *C. crenata*, being putative specific markers for *P. cinnamomi* resistance in these populations.

The QTLs reported in this study, should provide an extensive list of candidate genes for *P. cinnamomi* resistance, since the QTL intervals extend to as much as 10.3 cM. In the future, several resistance candidate genes will be identified in the QTL intervals from chestnut genome (in preparation). Nevertheless, some putative genes underlying these QTLs were identified, providing some clues about resistance genes involved in *P. cinnamomi* response. Regulation of gene expression, hormone signaling and synthesis of aromatic compounds, that may induce hypersensitive responses (Alkio et al. 2005), may be involved in *P. cinnamomi* resistance, as it has been suggested by previous studies of tree-*Phytophthora* interactions (Schlink 2010; Coelho et al. 2011; Serrazina et al. 2015, Chapter III). The strongest evidence that the QTLs identified here underlie resistance candidate genes,

corresponds to the mapping of CsPT0016 and CsPT0010 markers on LG_B and LG_K, respectively. These SSRs were developed from genes associated to pathogenesis (ERF family) and stress response (Zinc Finger family), which were differentially expressed in *C. sativa* transcriptome after root inoculation (Serrazina et al. 2015, Chapter III). Therefore, they are strong candidates for downstream studies and applications. In this context, the expression level of transcripts belonging to ERF and Zinc Finger families have been quantified for *C. sativa*, *C. crenata* and SC hybrids showing different phenotypes (Chapter IV).

Understanding the basic genetic structure of ink disease resistance will increase the accuracy of genomic selection for disease resistance. Since QTL effects vary across environmental and genetic backgrounds, additional populations will be genotyped and phenotyped to validate the QTLs here proposed. Additionally, in the near future, *C. sativa* x *C. crenata* populations will also be genotyped using a Genotyping-by-Sequencing (GBS) platform, which will increase the resolution of QTL mapping. The future availability of the chestnut genome sequence will allow the identification of all genetic elements within each *P. cinnamomi* resistance QTL and also the discovery of more molecular markers.

In summary, the new EST-SSRs, genetic linkage map and QTLs here presented constitute the first effort developed in an interspecific cross between *C. sativa* and *C. crenata* to map genomic regions associated with *P. cinnamomi* resistance and extending the chestnut genomic resources available. This constitutes a foundation for marker-assisted selection to be applied in the ongoing and future chestnut breeding programs worldwide.

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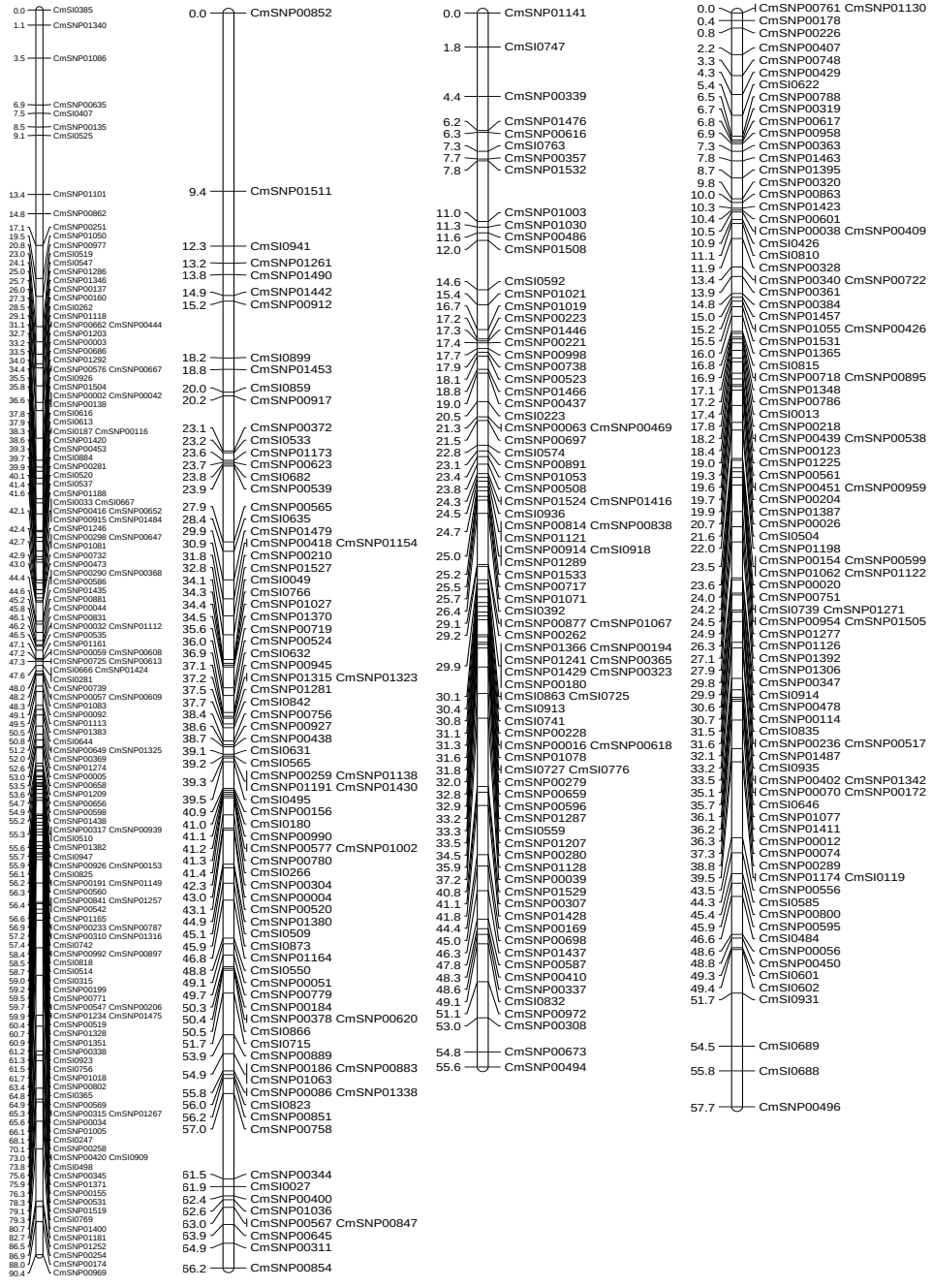
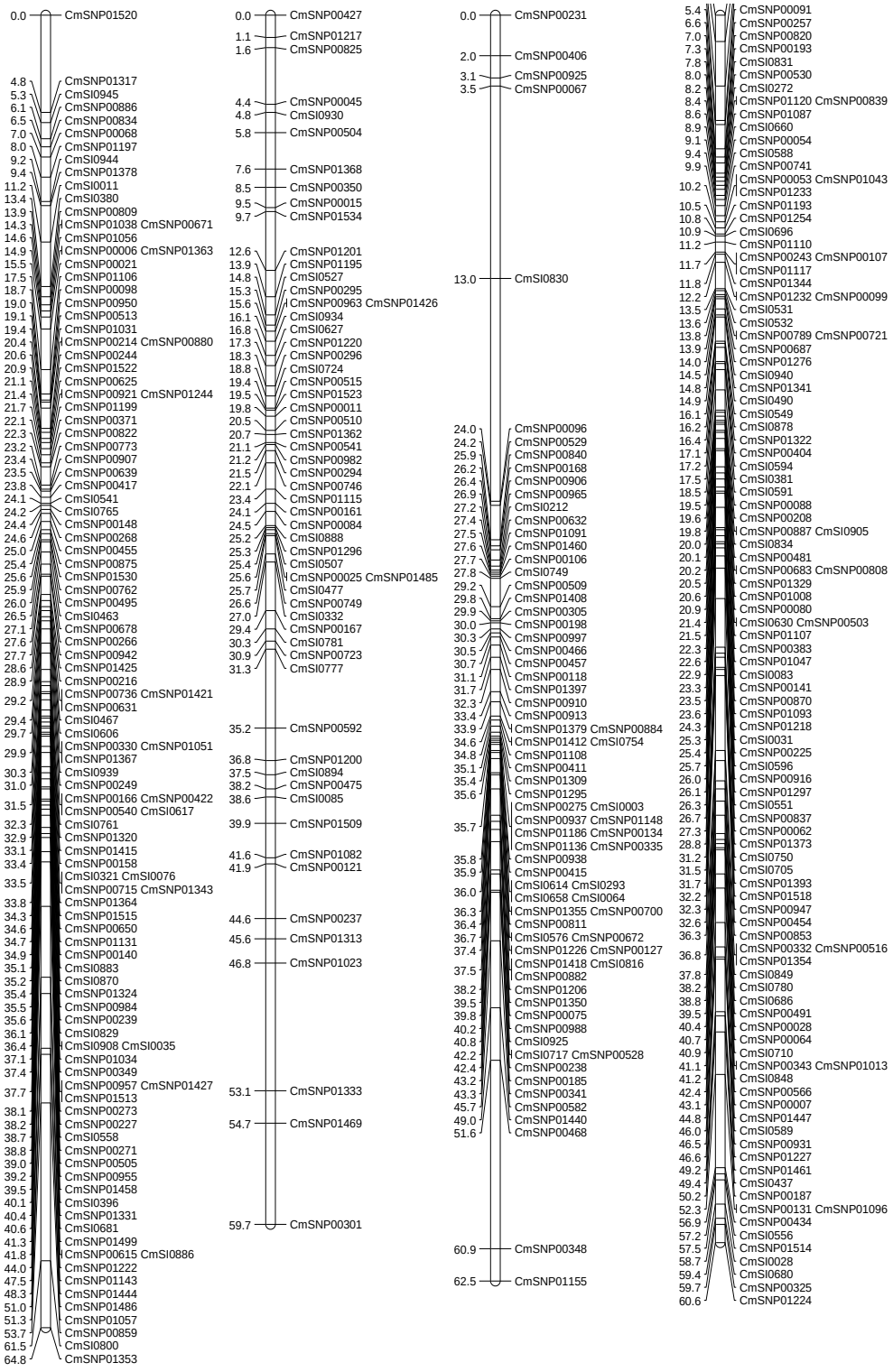
Supplementary material

Supplementary material 1. *C. mollissima* reference genetic map spanning 742.4 cM across 12 linkages groups [A-L (A-D:page 196, E-H:page 197 and I-L: page 198)] (Kubisiak et al. 2013).




0.0 CmSNP01520
4.8 CmSNP01317
5.3 CmSI0945
6.1 CmSNP00886
6.5 CmSNP00834
7.0 CmSNP00068
8.0 CmSNP01197
9.2 CmSI0944
9.4 CmSNP01378
11.2 CmSI0011
13.4 CmSI0380
13.9 CmSNP00809
14.3 CmSNP01038 CmSNP00671
14.6 CmSNP01056
14.9 CmSNP00006 CmSNP01363
15.5 CmSNP00021
17.5 CmSNP01106
18.7 CmSNP00098
19.0 CmSNP00950
19.1 CmSNP00513
19.4 CmSNP01031
20.4 CmSNP00214 CmSNP00880
20.6 CmSNP00244
20.9 CmSNP01522
21.1 CmSNP00625
21.4 CmSNP00921 CmSNP01244
21.7 CmSNP01199
22.1 CmSNP00371
22.3 CmSNP00822
23.2 CmSNP00773
23.4 CmSNP00907
23.5 CmSNP00639
23.8 CmSNP00417
24.1 CmSI0541
24.2 CmSI0765
24.4 CmSNP00148
24.6 CmSNP00268
25.0 CmSNP00455
25.4 CmSNP00875
25.6 CmSNP01530
25.9 CmSNP00762
26.0 CmSNP00495
26.5 CmSI0463
27.1 CmSNP00678
27.6 CmSNP00266
27.7 CmSNP00942
28.6 CmSNP01425
28.9 CmSNP00216
29.2 CmSNP00736 CmSNP01421
CmSNP00631
29.4 CmSI0467
29.7 CmSI0606
29.9 CmSNP00330 CmSNP01051
CmSNP01367
30.3 CmSI0939
31.0 CmSNP00249
31.5 CmSNP00166 CmSNP00422
CmSNP00540 CmSI0617
32.3 CmSI0761
32.9 CmSNP01320
33.1 CmSNP01415
33.4 CmSNP00158
33.5 CmSI0321 CmSI0076
CmSNP00715 CmSNP01343
33.8 CmSNP01364
34.3 CmSNP01515
34.6 CmSNP00650
34.7 CmSNP01131
34.9 CmSNP00140
35.1 CmSI0883
35.2 CmSI0870
35.4 CmSNP01324
35.5 CmSNP00984
35.6 CmSNP00239
36.1 CmSI0829
36.4 CmSI0908 CmSI0035
37.1 CmSNP01034
37.4 CmSNP00349
37.7 CmSNP00957 CmSNP01427
CmSNP01513
38.1 CmSNP00273
38.2 CmSNP00227
38.7 CmSI0558
38.8 CmSNP00271
39.0 CmSNP00505
39.2 CmSNP00955
39.5 CmSNP01458
40.1 CmSI0396
40.4 CmSNP01331
40.6 CmSI0681
41.3 CmSNP01499
41.8 CmSNP00615 CmSI0886
44.0 CmSNP01222
47.5 CmSNP01143
48.3 CmSNP01444
51.0 CmSNP01486
51.3 CmSNP01057
53.7 CmSNP00859
61.5 CmSI0800
64.8 CmSNP01353
0.0 CmSNP00427
1.1 CmSNP01217
1.6 CmSNP00825
4.4 CmSNP00045
4.8 CmSI0930
5.8 CmSNP00504
7.6 CmSNP01368
8.5 CmSNP00350
9.5 CmSNP00015
9.7 CmSNP01534
12.6 CmSNP01201
13.9 CmSNP01195
14.8 CmSI0527
15.3 CmSNP00295
15.6 CmSNP00963 CmSNP01426
16.1 CmSI0934
16.8 CmSI0627
17.3 CmSNP01220
18.3 CmSNP00296
18.8 CmSI0724
19.4 CmSNP00515
19.5 CmSNP01523
19.8 CmSNP00011
20.5 CmSNP00510
20.7 CmSNP01362
21.1 CmSNP00541
21.2 CmSNP00982
21.5 CmSNP00294
22.1 CmSNP00746
23.4 CmSNP01115
24.1 CmSNP00161
24.5 CmSNP00084
25.2 CmSI0888
25.3 CmSNP01296
25.4 CmSI0507
25.6 CmSNP00025 CmSNP01485
25.7 CmSI0477
26.6 CmSNP00749
27.0 CmSI0332
29.4 CmSNP00167
30.3 CmSI0781
30.9 CmSNP00723
31.3 CmSI0777
35.2 CmSNP00592
36.8 CmSNP01200
37.5 CmSI0894
38.2 CmSNP00475
38.6 CmSI0085
39.9 CmSNP01509
41.6 CmSNP01082
41.9 CmSNP00121
44.6 CmSNP00237
45.6 CmSNP01313
46.8 CmSNP01023
53.1 CmSNP01333
54.7 CmSNP01469
59.7 CmSNP00301
0.0 CmSNP00231
2.0 CmSNP00406
3.1 CmSNP00925
3.5 CmSNP00067
13.0 CmSI0830
24.0 CmSNP00096
24.2 CmSNP00529
25.9 CmSNP00840
26.2 CmSNP00168
26.4 CmSNP00906
26.9 CmSNP00965
27.2 CmSI0212
27.4 CmSNP00632
27.5 CmSNP01091
27.6 CmSNP01460
27.7 CmSNP00106
27.8 CmSI0749
29.2 CmSNP00509
29.8 CmSNP01408
29.9 CmSNP00305
30.0 CmSNP00198
30.3 CmSNP00997
30.5 CmSNP00466
30.7 CmSNP00457
31.1 CmSNP00118
31.7 CmSNP01397
32.3 CmSNP00910
33.4 CmSNP00913
33.9 CmSNP01379 CmSNP00884
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34.8 CmSNP01108
35.1 CmSNP00411
35.4 CmSNP01309
35.6 CmSNP01295
35.7 CmSNP00275 CmSI0003
CmSNP00937 CmSNP01148
CmSNP01186 CmSNP00134
CmSNP01136 CmSNP00335
35.8 CmSNP00938
35.9 CmSNP00415
36.0 CmSI0614 CmSI0293
CmSI0658 CmSI0064
36.3 CmSNP01355 CmSNP00700
36.4 CmSNP00811
36.7 CmSI0576 CmSNP00672
37.4 CmSNP01226 CmSNP00127
37.5 CmSNP01418 CmSI0816
CmSNP00882
38.2 CmSNP01206
39.5 CmSNP01350
39.8 CmSNP00075
40.2 CmSNP00988
40.8 CmSI0925
42.2 CmSI0717 CmSNP00528
42.4 CmSNP00238
43.2 CmSNP00185
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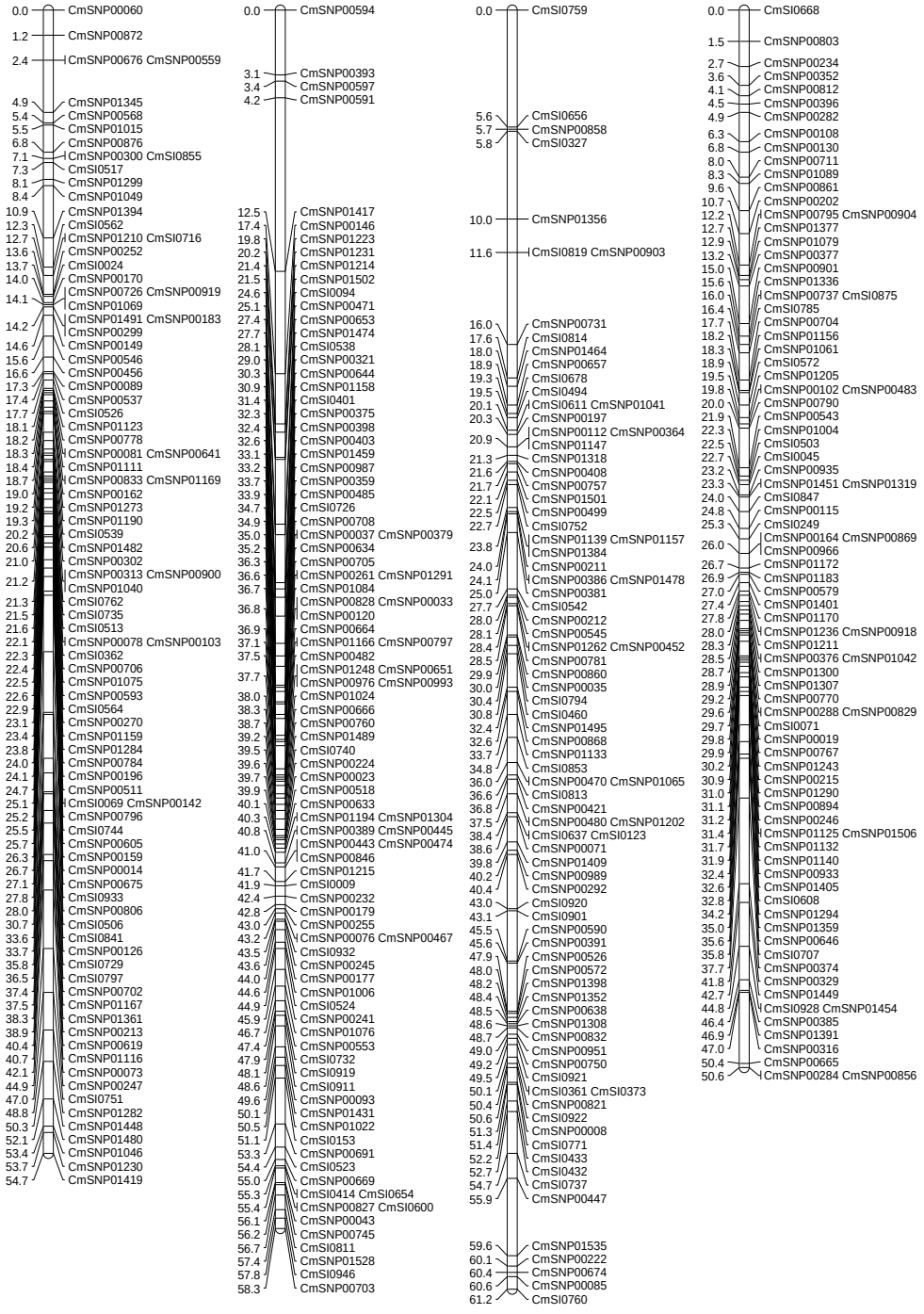
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Supplementary material 2. Characterization of 43 new EST-SSR markers: number of alleles (Na), expected heterozygosity (H_E), observed heterozygosity (H_O), repeat motif, primer sequences (5′−3′) and allele size average (Al. size av., in bp). Number of alleles expected and observed heterozygosities were estimated for 25 chestnut samples, comprising the four *Castanea* species used in Portuguese and the TACF breeding programs. Asterisk (*) indicates SSRs which amplify for the four species.

Locus name	Na	Ho	He	Repeat motif	Primer forward sequence (5′−3′)	Primer reverse sequence (5′−3′)	Al. size av.
CcPT_0001	2	0,33	0,28	(CT)12	CGAGGATTTC GAACCAGAGA	ATCACAATACA CGCGCAGAG	466
CcPT_0002*	1	0,00	0,00	(GTG)9	AGTTCTCCAC GAGGCTCAAA	TCCAAGCTGG AGAATCATCA	415
CcPT_0003*	7	0,43	0,74	(ATC)8	GGTGCCCAGA TTTACGAGAA	ATCGCTTGGA GTCACAGCTT	426
CcPT_0004*	6	0,27	0,64	(CT)10	GCTGCTTCAC AACCTTCCTC	GCAAGAGATT CCCTTTGCTG	375
CcPT_0005*	7	0,52	0,75	(CT)11	ACACATGGGG GTGTGAACTT	TTATGGGAAA CGGCATCTTC	175
CcPT_0006*	4	0,17	0,62	(TTC)7	CCTGTGAGGC TAAGAGAGCG	ACCACGTCGG TGCTTCTAGT	339
CcPT_0008*	5	0,29	0,77	(TCT)10	TCGTCCCCTT CTTCATCATC	ATATGGCCAA AAACCCATCA	586
CcPT_0009*	8	0,23	0,80	(TC)9	TTCCACCCAAT TGTTACCAC	GATGATGAAG AAGGGGACGA	316
CcPT_0010*	5	0,37	0,67	(GGT)7	ATCCATGAGT GAAAGCCACC	TGGAACAAGA AGCCTCGATT	503
CcPT_0011*	5	0,39	0,75	(CAC)7	TCATCCAAGA AGCCCTCAAC	TTCTGCCTCTT TTGTTGCCT	431
CcPT_0013*	11	0,70	0,87	(AG)11	AGTACGTAGT CGAAGAGAGA AGAG	AGTGAAGTTTT GTCGGGGTG	197
CcPT_0014*	4	0,25	0,70	(TC)7	AGGCGCATTC AAAGAAAGAA	AGCTGATCAA CTCTCGCCAT	172
CcPT_0015*	4	0,44	0,48	(CAA)7	AGTCTTTGGC GTCAGCAAAT	GCCCATCTGA AATCCAAAAA	429
CcPT_0019*	1	0,00	0,00	(TCA)6	CCCATTCACC ATCAATTTCC	TCGTGATGCC TATCAATCCA	228

Locus name	Na	Ho	He	Repeat motif	Primer forward sequence (5'–3')	Primer reverse sequence (5'–3')	Al. size av.
CcPT_0020	7	0,82	0,83	(AG)8	GAACTTGCTT GTTGAAGGGG	CGCCATAACT TCCTTTTCCA	497
CcPT_0021*	5	0,52	0,63	(CCG)6	GCATGCCCAT ACCCATTAAC	GGATGCAAAG GCTTTAGCTG	316
CcPT_0022	9	0,29	0,82	(TC)7	GCTGCCTTCA TTACTTCCCA	TGAGATTGTC GAGGGTTTCC	377
CcPT_0023*	3	0,35	0,49	(TGT)6	GGCATGAGAA GAAGCTGAGG	GAGTTTCCAA GAACCCACCA	472
CcPT_0024*	3	0,04	0,43	(TTG)6	GGGCTTGGGC TTTTTCTTAT	GCACCCAATT TCAATGAACC	502
CcPT_0025*	9	0,50	0,75	(TTG)6	GGGTCGGAAT ACATGTGACC	GCTTTGATCC AACCAACGAT	352
CcPT_0026*	1	0,00	0,00	(CAG)6	GTTCCGGACA AGAATGAGGA	CGGCTGAAAG GATCAAGAAG	204
CcPT_0028*	5	0,44	0,52	(TGG)6	TCCAATGCCC AAC	GTTTCCCTTGA TGGGTTTGA	200
CcPT_0030*	4	0,44	0,65	(GGT)6	TCGAGGCTTC TTGTTCCACT	GAATTGGTGG AGGCAGAAAA	488
CcPT_0031	4	0,10	0,53	(AAG)6	TCTTTTCCCAT TGCCATCTC	CCCCAAATTCT TTTCTGGGT	447
CcPT_0032*	6	0,62	0,77	(TTG)6-(TTG)6	TGGCCATATTT TCTCCAAGC	CCACCGCCAG TACATCTTTT	663
CcPT_0034	6	0,67	0,78	(AAAG)5	TTTCACTTTCT TCCCATGCC	CAATTTCAAAG CTTTTGGGTTC	399
CcPT_0035*	5	0,26	0,66	(TTTC)5	TTTCTTTGCTT CTTTTGGGC	ACGCTCCATT ACAGCTGCTT	229
CsPT_0003	10	0,44	0,87	(AGC)7	CGCCGTAGTA CTGCTGATGA	CTTCTCCCCC TAACCTCTCG	424
CsPT_0004*	7	0,57	0,77	(GAG)10	ACAGGAATTG GGATCCATCA	CACACCCTCT TGTTCCACCT	477
CsPT_0005*	3	0,40	0,58	(AG)8	GCTTTTGGTT GATTTGCGAC	TAAGCCCTGA GAACATTGGC	289
CsPT_0006*	10	0,48	0,85	(TC)9	CCTTGCTTCG CTCAGTCATT	GATCCGACCC GTTTGAGTTA	384
CsPT_0007*	9	0,70	0,82	(CT)11	AGTGAAGTTTT GTCGGGGTG	TCGAAGAGAG AGAAGAGGAA GAA	184

Locus name	Na	Ho	He	Repeat motif	Primer forward sequence (5'–3')	Primer reverse sequence (5'–3')	Al. size av.
CsPT_0008*	3	0,57	0,55	(ATG)9	ATGCCCGCAA GATTGTTTAC	GAACTTAGGT GGCTCAAGCG	354
CsPT_0010*	9	0,18	0,86	(TGT)6	CACCTCTCTCT TCACCTCCG	TCTCAAAGCT CGTTTCCGAT	497
CsPT_0011	4	0,46	0,38	(CT)7	CAGGTTTCTC CCAAATCCAA	CACCTCAAGG GTGATGGTCT	286
CsPT_0013	5	0,65	0,65	(GGT)6	CTCCAATCAAT AGCCCTCCA	TTACCCTGTA GCCCAACCTG	319
CsPT_0015*	5	0,39	0,63	(CTT)7	CTTTAGCGAT CTTGGCGAAC	CCCTTCATTTT TGCTGGATG	455
CsPT_0016*	2	0,22	0,36	(AAG)6	GATACTCGAT GGGGAGCAAA	TGTTGAGAAG ATGGCAGCAG	307
CsPT_0017	2	0,25	0,22	(CCA)6	GCCACAAGGT CTGGATTGTT	GCAACACTGG TTGTCAAAGC	500
CsPT_0018*	4	0,52	0,59	(AGC)6	GCGATTTAAC GCTCTTCGTC	CCAAGCCAAC TGCTCCTAAG	501
CsPT_0020*	4	0,40	0,64	(TCT)6	TCCGATCGAA GACGAACTCT	TTTGTGCCGTT AATGATGGA	263
CsPT_0021*	5	0,65	0,75	(GGT)6	TCTCTTGCATC ACCGTCAAG	GATCCGACCC GTTTGAGTTA	183
CsPT_0022*	7	0,56	0,63	(TTAA)5	TGTTTGCACTT AGAGCGGTG	TTCAATTTCCC GGATTCAAG	394

Chapter VI

Conclusions and future perspectives

Ink disease is the most destructive disease affecting European chestnut and it will be even more difficult to control in the future, due to climate changes (Thompson et al. 2014). Therefore, it is urgently necessary to better understand how chestnut plants respond to *P. cinnamomi* infection, in order to improve them for resistance, contributing for a sustainable and increased chestnut production.

The work presented in this thesis is a pioneer study focusing on the use of different approaches (genomics, phenomics and transcriptomics) to disclose *Castanea* response mechanisms to *P. cinnamomi*, providing new insights for the ongoing and future chestnut breeding programs worldwide. Particularly, this project constitutes a fundamental contribution for the Portuguese chestnut breeding program for resistance to ink disease. The major outcomes achieved include: 1) the increase of the number of individuals in the mapping population; 2) the phenotyping and genotyping of all progenies; 3) the selection of a set of genotypes as the most resistant to *P. cinnamomi*, which may open a market opportunity; 4) the description of a possible molecular mechanism of resistance to *P. cinnamomi* and 5) the development of unique molecular tools and genomic resources related with *Castanea* response to *P. cinnamomi* , such as ESTs and candidate genes, molecular markers, genetic map and QTLs.

In the first part of this thesis (Chapter II), a robust phenotyping methodology was developed, in order to obtain reliable results for the next stages of this research, such as QTL analyses and gene expression quantification. Since environmental variation was minimized and biological replicates were used, the phenotypic values estimated result mainly from genetic variation, increasing heritability of the traits and the robustness for QTL detection (Chapter V).

The variables measured in both inoculation tests used for phenotyping (root inoculation and excised shoots inoculation) were phenotypically and genetically strong correlated, mainly between the ‘Days of survival’ (root

inoculation) and 'Lesion Progression rate' (excised shoots inoculation). Therefore, Lesion Progression rate was used to phenotype the new chestnut progenies obtained from controlled crosses. Lesion progression rate was also the selected variable to perform QTL identification (Chapter V), because it followed a continuous variation pattern genotype-dependent, enabling the analysis of resistance in a quantitative manner.

The root inoculation test mimics the natural conditions of infection, being the most accurate method to determine the level of susceptibility towards *P. cinnamomi*. The variable 'Days of survival' was the most important indicator of chestnut resistance to the pathogen. Therefore, long survival was considered evidence of high resistance and accordingly, seven genotypes were selected as the most resistant. Some of these resistant genotypes were also inoculated with *Cryphonectria parasitica* in order to verify their susceptibility to chestnut blight. This assay revealed a hybrid from *C. sativa* x *C. mollissima* crosses as the most resistant to both diseases (Santos et al. 2016). These new genetic resources constitute an excellent deliverable of this research, potentially usefull as *P. cinnamomi* resistant rootstocks, since the market present a big deficit of chestnut genotypes with improved genetic resistance. Accordingly, those genotypes have been propagated, through *in vitro* culture, to be registered and eventually released to the market in the near future (Santos et al. 2016). Graft compatibility with the best Portuguese varieties has been tested and the results obtained so far are promising; nevertheless, further investigation is needed on chestnut graft compatibility. Additionally, a set of new candidate genotypes, obtained from controlled crosses performed in 2015, will be validated by root inoculation test hereafter.

Chestnut resistance to *P. cinnamomi* seems to be related with the ability of the host to circumvent the pathogen establishment and growth, as the results obtained in phenotyping (Chapter II) and transcriptomic (Chapter III and IV) approaches revealed. In the phenotyping assays, the confinement of the

lesion to the point of inoculation was observed in resistant genotypes, both in root or shoot inoculations. In fact, for the most resistant genotypes, lesions surrounding tissues dried, limiting the progression of the lesion. Nonetheless, histological studies may reveal other important features on circumventing pathogen development.

At the molecular level, the recognition of the pathogen is believed to trigger a hypersensitive response, which is characterized by the localized production of reactive oxygen species (ROS), leading to accelerated cell death and inhibition of pathogen spread (Mur et al. 2008; Coll et al. 2011; Stael et al. 2015). Results from RNA-Seq (Chapter III), showed that upon *P. cinnamomi* inoculation, *C. sativa* and *C. crenata* up-regulated genes involved in HR/HR recovery. Additionally, DEGs involved in the regulation of HR by SA were also identified in *C. crenata*. Similarly, gene expression analysis (Chapter IV) suggests an important role of HR, mediated by SA or other pathways, such as by vital protein degradation (via *Cast_RNF5*) or by breaking actin dynamics (via *Cast_Gnk2-like*).

The root transcriptome also revealed a predominance of down-regulated genes in *C. sativa* upon pathogen inoculation, suggesting that *P. cinnamomi* may regulate host transcriptional activity, which may allow the establishment and spread of the disease. Previous studies showed that oomycete pathogen effectors encode RNA silencing suppressors, manipulating specific physiological processes or signaling pathways to disrupt host immunity (Qiao et al. 2013; Pumplin and Voinnet 2013). Therefore, the isolation and sequencing of small RNAs from the pathogen and host are required to confirm their influence in chestnut-*P. cinnamomi* interaction.

The resistance candidate genes identified for European and Japanese chestnut transcriptomes revealed many common functional categories in their responses to the pathogen. Nevertheless, distinct host susceptibility may be explained by the differences obtained in DEGs, as well as variations in gene expression ratios and timing. Likewise, in gene expression analysis

(Chapter IV), a lower and delayed expression of the candidate resistance genes was found in *C. sativa*, opposing to the high expression levels of those genes in *C. crenata*, indicating that basal defense mechanisms may be involved in resistance to *P. cinnamomi*.

Overall, the results obtained from transcriptomic approach suggest that the resistance response may involve chemical (synthesis and secretion of anti-fungal proteins) and physical (strengthening of cell wall) barriers; PAMPs of *P. cinnamomi* may be recognized by specific receptors, triggering host immune response through transcriptional regulation; HR/HR recovery may be essential for a resistance response, limiting *P. cinnamomi* lesions; finally, hormone signaling may also play a key role in response and maintaining physiological conditions. Moreover, some molecular markers mapped on QTLs identified in this study, were developed from ESTs annotated as transcripts involved in the referred resistance pathways.

Allelic variation of resistance genes may explain the differences in constitutive expression observed between the contrasting phenotypes. Indeed, high levels of polymorphism were observed between *C. sativa* and *C. crenata*, which are the progenitors of mapping populations.

A total of 435 transcriptome-derived molecular markers showed polymorphism in SC population and were used to construct the first Euro-Japanese chestnut genetic map (Chapter V). Although an extensive genetic distance was obtained, it is essential to saturate this genetic map in order to increase the resolution of QTL mapping. Enhancing marker density will be possible by increasing the number of individuals and/or using new genotyping platforms, such as genotyping by sequencing (Elshire et al. 2011).

Even so, ten QTLs associated with *P. cinnamomi* response were mapped on five linkage groups. Those regions may underlie several genes involved in the response to the pathogen. In fact, two microsatellite markers, developed from candidate resistance genes (Chapter III), were mapped on the identified

QTL intervals, being strong candidates for marker assisted selection in the future. However, the list of genes underlying the QTLs obtained will only be completed when the chestnut genome sequence becomes available. At that time, additional molecular markers will be developed on QTLs and the early selection of genotypes with improved resistance, through molecular markers, will be implemented.

Since genomic and transcriptomic data lacks validation by functional analysis, genetic transformation of European chestnut (via somatic embryogenesis) or model plants, with clonal vectors carrying resistance candidate genes will be performed in the near future. Long survival of transgenic plants inoculated with *P. cinnamomi* would validate those resistance genes. Moreover, epigenetic and proteomic approaches would corroborate our hypotheses or raise new biological questions.

Only time will show how those different approaches will individually and collectively contribute to establish new improved chestnut populations, carrying European chestnut genetic background, capable of surviving under presence of *P. cinnamomi*. As a great genetic conservation was observed across *Castanea* species and also a similar response to *C. parasitica* and *P. cinnamomi* was revealed, the present breeding program would enable the introgression of genes for resistance to ink disease and blight together.

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